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Morphological and Genetic Variability Analysis in the *Rhipicephalus sanguineus* (Parasitiformes, Ixodidae) Portuguese Populations

Mestrado em Biologia Humana e Ambiente

Rúben Miguel Ângelo Rodrigues Simões

Dissertação orientada por:

Doutora Fernanda Rosa - Instituto Superior de Agronomia

Professora Doutora Deodália Dias - Faculdade de Ciências da Universidade de Lisboa

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Nota prévia

Devido ao facto do Inglês ser a língua científica universal, a presente dissertação de mestrado encontra-se escrita na língua inglesa.

As partes desta dissertação escritas em Português, nomeadamente os agradecimentos e o sumário não respeitam o novo acordo ortográfico.

As referências bibliográficas foram elaboradas segundo os parâmentros da revista científica *Parasites & Vectors,* uma vez que esta é uma das mais relevantes na área da parasitologia onde esta tese se enquadra.

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Abstract

Ticks are arthropods with medical and veterinary importance. In particular *R. sanguineus* constitutes a risk to public health, being responsible for the transmission of several pathogens, namely *Ricketsia conorii,* the etiologic agent of Mediterranean spotted fever. This tick is very frequent in Portugal that currently presents one of the highest rates of incidence of tick borne diseases in Europe.

Ticks belonging to genus *Rhipicephalus* are extremely difficult to identify morphologically, due to the high level intraspecific variability. Ticks from the *R. sanguineus* group are associated with controversy, once the species identification and distinction are sometimes difficult due to their morphological similarities specialy between *R. sanguineus* and *R. turanicus*, which is a particularly challenging task. Portugal is not indifferent to the taxonomic issues between this two species, since the results obtained in previous studies differ, and there is much disagreement around their taxonomic classification.

In order to promote more consistent taxonomic reconstructions, morphological studies should be applied together with biological and molecular approaches. It is in this context that this study appears, once it combined a morphological study, in which several quantitative and qualitative variables were considered and studied through a-statistic analysis and simultaneously a rigorous morphological analysis was conducted on several specimens of Portuguese *Rhipicephalus sanguineus*. A representative sample from each clusters obtained were selected for a genetic study using 12S and 16S molecular marker.

Results revealed the presence of great morphological variability in the Portuguese populations of *R. sanguineus* and also the existence of some interesting genetic variability. Although not enough to justify the classification as different species. However phylogenetic analysis highlight the grouping in separate tree branches, suggesting the possibility of the beginning of a speciation.

Keywords: Molecular analysis, morphological characterization, *Rhipicephalus sanguineus.*

Súmario

As carraças são artrópodes, da classe Arachnida, ectoparasitas obrigatórios e apresentam grande relevância médica e veterinária, devido à sua acção hematófaga e à sua capacidade de transmitir vários patogéneos, nomeadamente vírus, protozoários, helmintes e fungos. São consideradas o segundo vector mais importante na transmissão de agentes causadores de doenças humanas a seguir aos mosquitos, sendo responsáveis por mais de 100000 casos de doença humana em todo o mundo. De igual modo são, os vectores mais importantes em termos de transmissão de patógenios causadores de doença a animais domésticos e silvestres, e consequentemente responsáveis por grandes danos económicos.

Dentro das várias espécies de ixodídeos existentes, o género *Rhipicephalus* da família Ixodidae é o que tem maior distribuição mundial, sendo simultaneamente um dos mais controversos, pela grande semelhança interespecífica evidenciada pelas espécies que agrupa. As espécies envolvidas, caracterizam-se ainda pela capacidade de parasitar uma grande diversidade de hospedeiros vertebrados e pela sua eficácia como vectores de diversos agentes patogénicos. Uma das questões dentro deste género está relacionada com a distinção de duas espécies nomeadamente *R. sanguineus* e *R. turanicus* que, devido a ausência de características morfológicas, permitam a sua distinção óbvia.

Em particular, as carraças da espécie *R. sanguineus* constituem um risco para a saúde pública, uma vez que são responsáveis pela transmissão de uma grande diversidade de agentes patogénicos causadores de doenças a cães e humanos. As doenças mais graves em cães são a babesiose, causadas por *Babesia canis* e a erliquiose monocítica, causada por *Erlichia canis*. No que diz respeito aos humanos a doença mais grave é a febre botonosa ou escaro-nodular, transmitida pela bactéria *Rickettsia conorri*. Esta ultima é uma doença de declaração obrigatória em Portugal, apresentando uma taxa de incidência de 9,8/105 habitantes, uma das mais elevadas da Europa.

Esta incidência deve-se ao facto de Portugal exibir condições ecológicas como vegetação adequada, grande variedade de hospedeiros e condições climáticas que propiciam a adaptação de carraças e dos agentes patogénicos por elas transmitidas. Acredita-se ainda que as alterações climáticas, que se têm verificado e que se irão intensificar nas próximas décadas, deverão contribuir para o agravamento desta situação, pois o aumento da temperatura média favorece a proliferação destes vectores.

Portugal não é indiferente às questões taxonómicas existentes no género *Rhipicephalus*, em particular à distinção das espécies *R. sanguineus* e *R. turanicus*. Estudos conduzidos anteriormente indicavam a existência destas 2 espécies em Portugal mencionando que *R. sanguineus* se encontrava associado ao cão e que *R. turanicus* se encontrava associado a ruminantes. No entanto, estudos posteriores relevaram que estas duas espécies são morfologicamente idênticas e não distinguíveis do ponto de vista genético, em Portugal.

Uma vez que estas duas espécies poderão estar associadas a capacidades patogénicas e vectoriais distintas e considerando, que Portugal possui características eco-ambientais que favorecem a manutenção e a proliferação de carraças e dos agentes patogénicos por elas transmitidas, é relevante em termos de saúde pública, a compreensão desta questão e conseguir caracterizar as populações portuguesas de *R. sanguineus sensu lato*.

Sendo as espécies do género *Rhipicephalus* extremamente difíceis de identificar morfologicamente, devido à elevada variabilidade intraespecífica, os estudos morfológicos devem ser acompanhados de estudos moleculares, de modo a promover reconstruções taxonómicas mais consistentes e é neste contexto que este estudo surge. Assim, foi o principal objectivo desta dissertação avaliar e caracterizar morfologicamente através do estudo estatístico de variáveis quantitativas e qualitativas, o que levaram à formação de clusters qualitativos, quantitativos e morfológicos A partir destes clusters foi possível avaliar as diferenças que os caracterizavam e quais as variáveis que mais contribuíam para a sua distinção. Outro objectivo foi inferir se a variabilidade morfológica correspondia também a variabilidade genética. Para esse objetivo, vários espécimes representantes dos clusters formados foram selecionados para um estudo genético recorrendo os marcadores moleculares (12S e 16S).

Os resultados obtidos revelaram a presença de uma grande variabilidade morfológica, formando 8 clusters morfológicos nos machos, e 5 nas fêmeas, os quais apresentam várias diferenças entre si, especialmente em termos das placas espiraculares nos machos e da abertura genital nas fêmeas. Os resultados obtidos neste estudo vieram ainda confirmar que as placas espiraculares nos machos e a abertura genital nas fêmeas são, de facto, as estruturas mais adequadas para diferenciar *R. sanguineus* de *R. turanicus*. Uma vez que se verifica que os machos *R. turanicus* possuem espiráculos mais largos e curtos e os machos de *R. sanguineus*, apresentam espiráculos mais finos e longos; as fêmeas de *R. sanguineus* apresentam aberturas genitais em forma de U aberto, com os escleritos bem afastados entre si,

as fêmeas de *R. turanicus* apresentam abertura genital em forma de U fechado com os escleritos próximos um do outro, como havia sido previamente descrito na literatura.

Os resultados moleculares revelaram a existência de variabilidade intraespecífica mas não suficientemente elevada para justificar a classificação em 2 espécies distintas. Foi ainda possível concluir que todos os haplotipos obtidos neste estudo se encontram inseridos no grupo *R. sanguineus* T2, e são genética e filogeneticamente distintos dos outros 3 grupos filogénicos previamente descritos (*R. sanguineus* T1, *R. sanguineus sensu lato* and *R. turanicus*). Os resultados suportam ainda a hipótese apresentada em estudos anteriores, que existem diferenças genéticas consideráveis entre a linhagem norte associada a clima tropical, e a linhagem sul associada a clima mais moderado.

É ainda digno de destaque que alguns haplotipos obtidos com o marcador 16S, quando analisados filogenicamente surgem agrupados num ramo isolado, formando um de mini-clade, sugerindo que está a ser observado é muito provavelmente o início de um processo de especiação. No entanto, estes estudos deverão prosseguir no sentido da maior clarificação desta problemática.

Palavras-chave: *Rhipicephalus sanguineus, Rhipicephalus turanicus*, análise molecular, análise filogénica, caracterização morfológica.

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List of Abbreviations

ANOVA - Analysis of Variance

BOLD - Barcode of Life Data Systems

CYTB - Cytocrome B

COI or COX1- Cytocrome Oxidade I

COIII - Cytocrome Oxidade III

CA- Correspondence Analysis

DNA- Deoxyribonucleid acid

HCA - Hierchical Cluster Analysis

ISA- Instituto Superior de Agronomia

ITS1- Internal Transcribed Spacer 1

ITS2 - Internal Transcribed Spacer 2

LAS - Leica Application System

mtDNA - Mitochondrial Deoxyribonucleid acid

MEGA - Molecular Evolutionary Genetics Analysis

NJ - Neighbour Joining

PCR - Polymerase chain reaction

N. – Number

RNA - Ribonucleic Acid

rDNA - Ribossomal Deoxyribonucleid acid

rRNA - Ribossomal Ribonucleic Acid

R. sanguineus - *Rhipicephalus sanguineus*

R. sanguineus T1 - *Rhipicephalus sanguineus* Type 1

R. sanguineus T2 - *Rhipicephalus sanguineus* Type 2

R. sanguineus af- *Rhipicephalus sanguineus sensu lato*

R. sanguineus s. l. - *Rhipicephalus sanguineus sensu lato*

R. sanguineus s. s. - *Rhipicephalus sanguineus sensu stricto*

R. sanguineus Int - *Rhipicephalus sanguineus* Intermediate

R. turanicus - *R. Turanicus*

R. pusillus - *R. Pusillus*

SEM - Scanning Electron Microscopy

Std. Deviation - Standard Deviation

Std. Error - Standard Error

HSD – Tukey Honestly Significant Difference

USA - United Stades of America

US Dollars - American Dollars

WHO -World Health Organization

1. Introduction

1.1 Historic background of *Rhipicephalus sanguineus*

1.1.1 Historic perspective

The historic origin of *R. sanguineus* can be divided into two segments, its history and the history of the knowledge concerning itself. One of first mentions to ticks was made by Aristoteles in his famous *Historia Animalia*, where he described certain aspects of the ticks habits and host relations. Later the Roman Pling wrote a mixture of facts regarding the habits of ticks in his book *Historia Naturalis.* This subject was also treated by Cato in *The Agriculture* [1].

Despite the early realization that ticks are ectoparasites in mammals, few knowledge regarding ticks was added until the eighteen century, when Linnaeus developed the nomenclature system and contributed to the current taxonomic scheme of animalia, wich is still being applied in modern times. In 1746, the first tick was described and included in *System Naturalis* with the descriptions of 24 species of the genus *Accarus*. Posteriorly, in 1795, Latrielle divided the genus *Accarus* into 11 new genera that preceded the current taxonomic classifications. The early 1900's saw attempts to investigate the anatomy of ticks exemplified in various papers of Bonnet, Samson, Robinson, as well as the biological studies carried on by Bishop in the USA and Loundsby in Africa [1].

One of the most significant discoveries, which lead to further investigations, occurred in 1893 by Smith and Kilbourne, who identify the pathogen responsible for the Texas Fever in humans, *Babesia microti*, whose transmission was made by a tick, *Boophilus annulatus.* It was the first moment in which the transmission of a protozoan by an arthropod was confirmed. After that, the field of taxonomy evolved with a large number of papers by Cooley, Hoogstraal, Delpy, Theiler, Posmerantzev and Roberts, the biological field was also the subject of an intense study and in more recent decades, pathological studies has emerged from the identification of ticks as vectors in a great diversity of pathogens worldwide.[1].

Regarding the particular history of *R. sanguineus*, it is believe that this species existed for a very long time in Egypt; this conclusion is supported by the recent finding of a dog mummy infected by ticks, in a tomb surrounding a Roman fortress. This discovery also raises an interesting question on the origin of dogs and their ticks. During the Roman Empire and its colonization, which started about 270 B. C., the Mediterranean witnessed a serious of relevant historic events, namely the intense waves of migration that occurred during and after the fall of the Roman Empire.These migration might have been a crucial factor to the dissemination of dog ticks around the Mediterranean region. Indeed, the Roman Empire expanded for more than four centuries and in maximum of its extension reached all countries surrounding the Mediterranean Sea, as far as Turkey, Lebanon, Iran, Arabia in the East, Germany and Britain in the North of Europe. So, considering that *Rhipicephalus* is typically an African genus, the most probable hypothesis, explaining the introduction of the *R. sanguineus* in Europe, is that at a certain point of time, this occurrence took place as result of the migration from people and their dogs from North Africa, during the Roman Empire or soon after his collapse [2].

So, this hypothesis may explain the introduction and dissemination of *R. sanguineus* in Europe, however R. *sanguineus* species is considered as the tick with a wider geographical distribution worldwide and currently it is established in North, Central and South America, Europe, Africa, Asia and Oceania [3]. This broad distribution is possible due to another historic occurrence, namely the globalization, which has increased the mobility of pets, in particular that has occurred since the fifteenth century, which allowed this species expansion [4]. In the last few decades the number of pets (particularly dogs) has increased considerably in many countries, which also contributed for the establishment of this tick species in several geographic locations [5].

1.1.2 Taxonomy

Ticks are an ancient lineage with origin in the cretaceous, about 100 million years ago, several data indicate that the two most important families of ticks existent today had differentiate by that time [6].

These parasites are obligatory hematophagous mites are included in the suborder Ixodida (phylum Arthropoda, class Arachnida, subclass Acari, order Parasitiformes), containing 3 families: Argasidae, mainly characterized by the absence of dorsal shield, being designated as

soft-bodied ticks; Ixodidae with dorsal shield, being designated as hard-body ticks; and Nuttalliellidae that have in *Nuttalliella namaqua* its sole representative, is a rare species only known in South Africa, that has intermediate characteristics of the other two families [6, 7].

A total of 896 species of ticks are recognized currently; the Ixodidea family contains 702 species distributed in 14 genera, including *Rhipicephalus*, originally described by Koch in 1844, this genus comprises 82 species including 5 from the former genus *Boophilus*; the Argasidae family contains 193 species and the family Nuttalliellidae is monotypic, therefore contains only one species [6].

R. sanguineus belongs to the subfamily Rhipicephalinae in the Metastriate (one of the two lineages of hard ticks), within the family Ixodidae. However the specific taxonomic classification of *R. sanguineus* is an ongoing debate [8].

Because of this, the genus *Rhipicephalus* was divided into eight groups or complex according to their morphological similarities: R. *appendiculatus, R. cliffordi-senegalensis, R. evertsi, R. kochi, R. pravus, R. sanguineus, R. simus* and *R. tricuspis*. The species of the *R. sanguineus* complex assume *R. sanguineus sensu stricto* as the basis of their taxonomic entity [9].

Although taxonomic status of *R. sanguineus* is very controversial, it can be said that at least 11 species are considered in this complex, namely: *R. sanguineus s.s.* (Latrielle, 1806) *R. bergeoni*, (described by morel and Balis in 1976) *R. camicasi* (described by Morel, Rodhain and Mouchet in 1964) *R. guilhoni* (described by Morel and Vassiliades in 1963), *R. leporis* (described by Pomerantsev in 1946), *R. moucheti* (described by Morel in 1964), *R. pumilio* (described by Schulze in 1935), *R. pusillus* (described by Gil Collado in 1938), *R. schulzei* (described by Olenev in 1929), *R. sulcatus* (described by Neumann in 1908) and *R. turanicus* (described by Pomerantsev in 1940) [7]. A representative taxonomic tree of *R. sanguineus* group is present in figure 1.

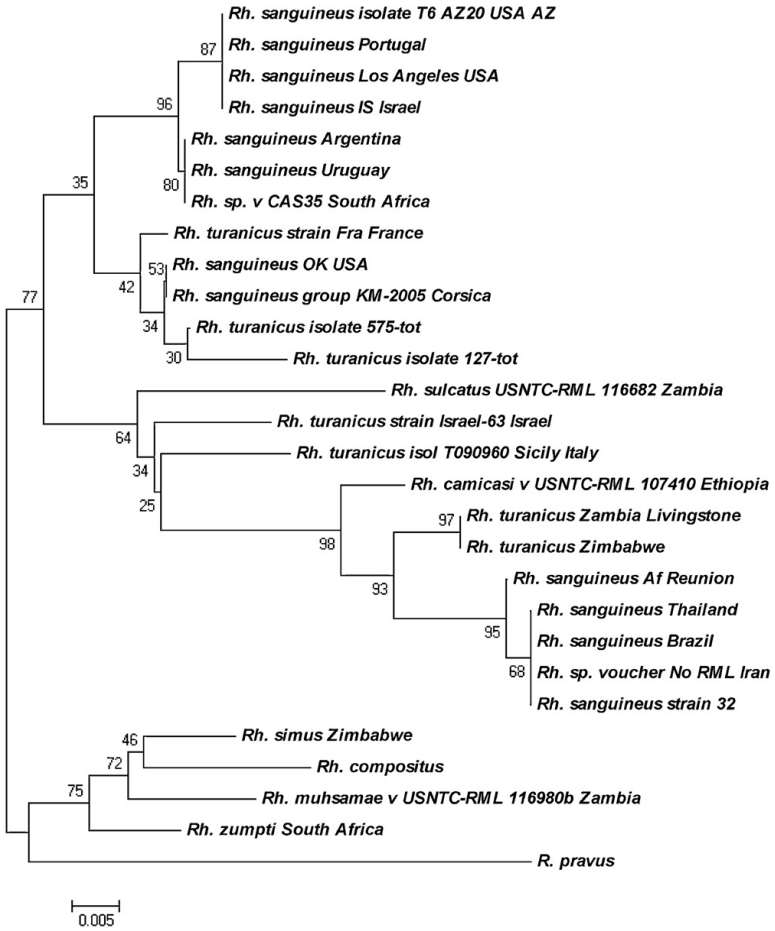


Fig. 1: Taxonomic Tree of the *R. sanguineus* group: NJ phylogenetic tree of 12S partial sequences (287bp). Numbers next to the branches represent percentages of replicate trees (out of 1000) in which associated taxa clustered together in the bootstrap test [10].

R. sanguineus species was originally described by Latreille in 1806 as *Ixodes sanguineus* and later placed in the genus *Rhipicephalus* by Koch, in 1884. Posteriorly, in 1911 Newman was the first to critically analyze this group of species and he was responsible for the synonymization of several species. A second attempt to revise this group was performed by Zumpt some years later. Despite that, only in 1940, through the studies of Pomerantsev the reference "sanguineus" was assigned to the genus *Rhipicephalus*, who provided this name to mention the ticks found on dogs in Mediterranean, because the original specimen described by Latreille was lost and his exiguous description did not provide an appropriate overview of the species [11]. This Pomerantsev pioneer idea was the basis of the contemporary concept of the group *R. sanguineus* originated by several authors, such as Hoogstral, Feldman, Morel and Filipova [12].

Despite that, *R. sanguineus* species is surrounded by very little consensus. Controversy begins after the attribution of an African origin by some authors in the opposition to others that

proclaim its Mediterranean origin. The fact that the genus *Rhipicephalus* is considered typically African causes the first theory to be more acceptable [8].

Simultaneously, it was not believed that the *R. sanguineus* ticks, distributed worldwide, represented a single species, these hypothesis found support in a study of the genital aperture of specimens in *R. sanguineus* showed that several species could be discriminated [13] . At the moment, it has been proposed the existence of two strains of *R. sanguineus*, one predominantly associated with the dog, mostly endophilic, and a "wild race" that parasitized wild carnivores [14].

It was then suggested that *R. bergeoni* should be removed from the R. sanguineus group, because it shares more affinities with *R. appendiculatus*. However the main taxonomic issue within this group is the morphological variation in the species *R. sanguineus* and *R. turanicus* [15]. Several authors consider both *R. sanguineus* and *R. turanicus* to be valid species and proposed several morphological features that allow the separation of both species [15–17].

Nevertheless even considering these morphological features, the separation of both species is a very difficult task; such difficulties arise because the species within the group do not have sufficient discriminating features between the different morphological characteristics which is related to a great intraspecific variability [18].

More recent studies use morphological and molecular evidence to understand the morphology of ticks and several phylogenetic studies were performed with members of the subfamily Rhipicephalinae. These studies brought new knowledge that lead to significant alterations to the traditional phylogenies, based only in morphological characters.

In that context, several studies were performed, namely with the molecular marker 16S, which demonstrated some significant genetic differences alongside the morphologic ones, between *R. sanguineus* and *R. turanicus* [19].

Later, using the molecular markers COI and 12S, it was found that genus *Rhipicephalus* was paraphyletic with respect of the species of the genus *Boophilus* [20]. This result associated with the findings of other studies using the molecular marker 16S [21] and also ITS2, COI and 12S [22] contributed to synonymize the genus *Boophilus* within the genus *Rhipicephalus*.

However this inclusion within the genus *Rhipicephalus* is still not accepted by many authors, despite the molecular evidences, due to several morphological and physiological differences within both genus, namely the *Boophilus* displays oval spiracular plates, does not present festoons and only use one host to complete its life cycle [23].

Despite all these echoes regarding this group, phylogenetic analysis of the *R. sanguineus* complex, using concatenated amino acid sequences of 13 protein-coding genes by three different computational algorithms (MP, ML and Bayes) provided molecular support that R. *sanguineus* represent indeed a species complex [24].

It is also noteworthy that the genus-level taxonomy of the family Argasidae is even more uncertain than the Ixodidae, at the species level, there are two factors responsible for such uncertainty; first the lack of adequate guidelines based on stable morphological features and second the fact that high biodiversity present by that family has been underestimated regarding the taxonomic keys [25].

Currently the main taxonomic issue within the *R. sanguineus* group is not how to separate *R. sanguineus* and *R. turanicus*, but to recognize the "morphological limits" that define each species and to accommodate large numbers of specimens within such a range of variation if necessary, new species should be erected and defined, but always within an adequate framework of morphology, ecology and DNA traits. Local or even regional variations of these ticks are frequent and they are not an excuse for species erection [11].

1.2 Biology of *Rhipicephalus sanguineus*

1.2.1 Morphological Characterization: Identification and Sexual Dimorphism

The ticks external structure is composed by 3 major regions, the anterior called gnathosoma also kwown as capitulum, the posterior idiossoma, usually called body and the legs. The capitulum is formed by the basis capitulum, whose function is to attach the body to the four segmented palps, chelicerae and hypostome that contains rows of teeth. The idiossoma is divided into two regions, the anterior called podossoma, containing 4 pairs of legs and the genital aperture in females, and the posterior denominated opistossoma bearing the anal aperture, the festoons grooves and the spiracular plate. Finally, the legs are sub-divided into 6 segments namely: trochanter, femur, tibia, tarsus, pre-tarsus and coxae, being this last one responsible for connecting the legs to the body. Tarsus in the first pair of legs contains the Haller's organ. Little is known about this structure but is believed that it is a sensory organ used for detecting heat and several odors in questing new hosts [6, 26].

The species of ticks *R. sanguineus* in particular are characterized by being small or medium-sized, red-brown in coloration, have elongated body-shape, indistinct anal opening, usually lack staining ornaments, short palps, presence of eyes and festoons. The base of the dorsal basis capituli presents hexagonal shape, coxae I is deeply cleft, the spiracular plates are located near coxae IV, which in males are shaped like commas and in females are oval shaped, shorter and wider than in males (fig.2) [3, 27, 28].

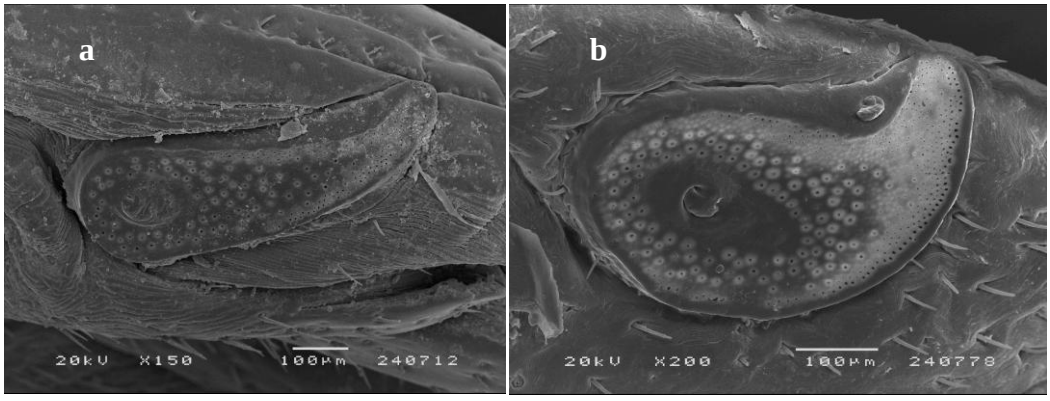


Fig. 2: Differences between the male and female spiracular plate in *R. sanguineus*: (a) presents the male spiracle, this presents comma shape and is thinner and narrower upwards **(b)** presents the female spiracle this present oval shape and is wider and larger than the male´s.

These difference between the shape of the spiracular plates among males and females is not only due to the overall wider body presented by females but also the result of different physiological necessities exhibited by these gender such as, the digestion of larger blood meals, egg production, excretion and other metabolic process. It is also believed that several ecological factors, for instance climate, also have an effect on the spiraculars plates form and size [29].

The first stage of development is the egg, which is characterized by being small, spherical, and dark brown. The hatched larvae are small, measuring on average 0.54 mm by 0.39 mm in length and width, respectively, and have only three legs on each side of their body. The next stage, the nymphs, have four pairs of legs, and they are similar to adults, (particularly to females due to the incomplete scutum they show) except that they have smaller dimensions on average 1.3 mm long by 0.60 mm wide and do not have genital opening, because they are immature stages and do not exhibit porose areas [3, 8].

The adult matches the phase of sexual maturity; in this phase the ticks has four pairs of legs and also sexual dimorphism (fig. 3): Males are flattened dorsal-ventrally and have dimensions in the order of 3 mm long and 1.5 mm wide, they present a complete dorsal shield, adanal plates, accessories shields on thethe ventral face, and also comma-shaped spiracles and a reddish-brown coloration as well as punctuations of variable size distributed in the dorsal region. On the other side, females are larger in size and present incomplete dorsal shield allowing them to ingurgitate more than males. Females have oval spiracles with a shorter tail, and also present porose areas on the dorsal surface of the basis capitulli with connection to nerve endings that have chemical-tactile functions, it appears that after the engorgement, the differences are accentuated, because after this process the females swells up to 11.5 mm long by 7.5 mm wide and the part of their body that is increased in size becomes blue-gray [3, 27]. There is also a difference in terms of the hypostomal teeth; males present 6-7 and females present 8-10 [30].

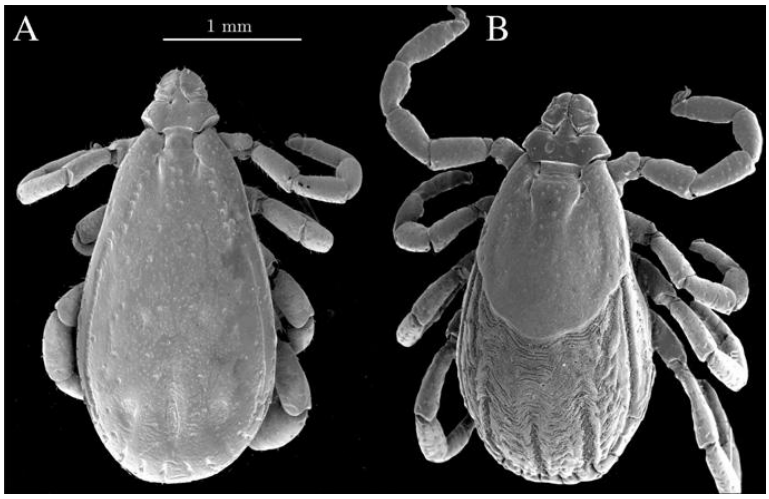


Fig. 3: Differences between the *R. sanguineus* male and female: Scaning electron micrographs of adults' *R. sanguineus,* dorsal view, illustrating basic features of the genus. (A) Female. (B) Male. It is noteworthy the presence of incomplete dorsal shield in the female and of porose areas in the basis capituli, something that does not occurs in the male specimen, once it presents a complete dorsal shield and the absence of porose areas [11].

R. sanguineus, from the morphological point of view, is very similar to *R. turanicus*, despite that there are several morphological structures, that can be used as a tool to differentiate both species, namely examining the females genital aperture, once *R. sanguineus* presents a circular anterior edge an wider than deep cup and *R. turanicus* exhibit a narrower U-shape aperture with higher sclerites (fig. 4) [31].

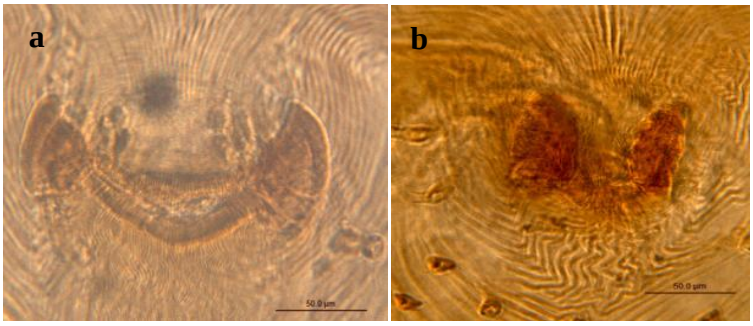


Fig. 4: Differences between the female genital apertures *R. sanguineus* (a) vs *R turanicus* (b): (a) this structure in *R. sanguineus* it exhibits a broad U-shape and a wider opening, **(b)** displays this structure in R. turanicus, it exhibits a V-Shape and a narrower opening

Besides the genital aperture, there are also key differences in terms of the spiracular plates and the adanal plates, namely, the tail of the spiracular plate are thinner in *R. sanguineus;* less than half of the adjacent festoon, by opposite that isn't observable in *R. turanicus.* However this observation, is not as evident in females, also *R. sanguineus* presents rounder adanal plates termination, and *R. turanicus* is associated with a sharper termination of the adanal

plates. In relation to females the major difference is in fact genital aperture: *R. sanguineus* displays a broad U-shaped aperture and in turn *R. turanicus* presents a V-shaped aperture. It was also noted that the cervical grooves in males are longer in *R. sanguineus*, and also that the termination of the females scutum is more linear in *R. sanguineus* than in *R. turanicus* [15].

However, it is believed that the most differentiating morphological traits for this two species, are the adanal plates for males, the genital aperture for female, and the spiracular plates for both genders [17]. Posteriorly, it was noted that the intraspecific morphological variation among ticks of *R. sanguineus* and *R .turanicus* in females is translated in differences in the female scutum pattern, genital aperture shape and spiracular plates, and in males it is translated in spiracular plates and in the adanal plates shape (fig. 5) [32]. Still, it is important to note that hibridation between *R. sanguineus* and *R. turanicus* is possible and, in that case, adanal plates are no longer on effective separation criteria between these two species [33].

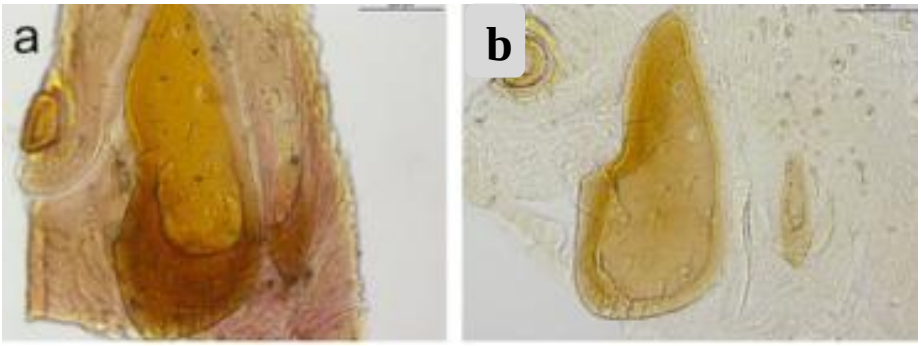


Fig. 5: Differences between males' adanal plates *R. turanicus* (a) vs *R sanguineus* (b): (a) presents this structure in *R. turanicus*. It presents a sharp termination; (d) presents this structure in *R. sanguineus*. It presents a rounder termination, and slightly smaller dimension than what occurs in *R. turanicus* [32].

Although several authors defend different points of view, the main morphological differences between *R. sanguineus* and *R. turanicus* are: in male, the ending of the spiracle tail is inferior or equal to half of the adjacent festoon in *R. sanguineus*; the ending of the spiracle tail is superior to half of the adjacent festoon, in *R. turanicus*; in females, *R sanguineus* exhibits a genital opening in the shape of an open U with sclerites slightly wider than lower and far apart from each other; *R. turanicus* females show a genital opening in the shape of a close U, with sclerites slightly higher than wider and closer to each other; the ending of the spiracular tail is higher and narrower in *R. sanguineus* while in *R. turanicus* these structure are wider and shorter (fig. 6) [34].

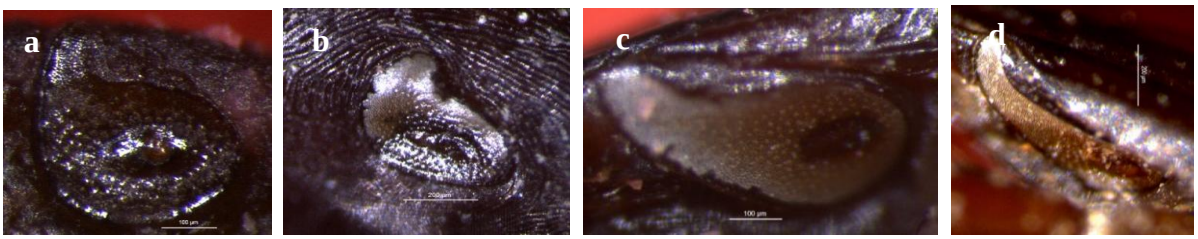


Fig. 6: Differences between the males and females spiracular plates *R. turanicus* (b) and (c) vs *R sanguineus* (a) and (d): (a) Represent the spiracular plate of *R. sanguineus* in a female and **(d)** the same structure in a male. The spiracular tail is higher and narrower; **(b)** Spiracular plate in *R. turanicus* in a female and **(c)** the same structure in a male. The spiracle tail is wider and shorter.

Both the immature *R. sanguineus* and *R. turanicus* have less morphological variation than adult forms, the fact that immature female forms do not possess genital aperture contributes for that occurrence and simultaneously the other distinctive morphological characteristics are less observable, at these stages [11].

1.2.2 Lifecycle

The *R. sanguineus* species is a three-phase type tick, each stage of development (excluding the egg) larva, nymph and adult feeds on a different host, which may be the same, in certain circumstances (fig 7) [12, 27]. From the ethological point of view, it is endophilic (adapted to indoor living), however is also able to survive in outdoor environment, according to its survival necessities and the surrounding environments [35].

Ticks spend most of their cycle away from the host [36]. However the successful attachment to a host, is crucial for its survival and perpetuation. When seeking for a host, the *R. sanguineus* is a hunter, although it can also adopt the ambush strategy, this behavior pattern displayed is result of its close relation with the domestic dog through its evolutionary history [35].

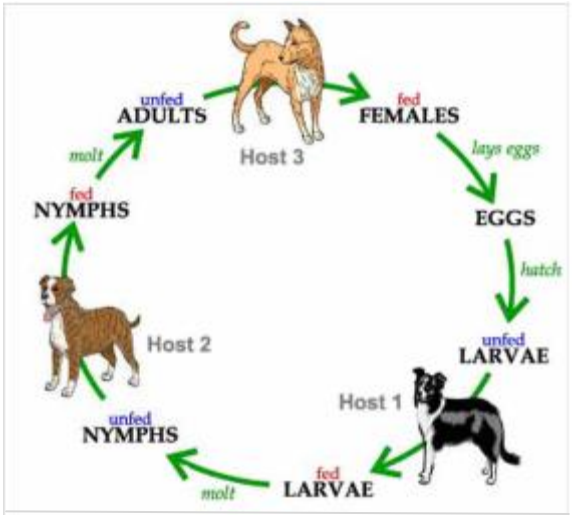


Fig. 7: Life Cycle of *R. sanguineus:* The complete life cycle, of a 3 host tick from egg to adult [37].

After a host is found, the attachment process follows. *R. sanguineus* can attach everywhere on the dog, it was commonly believed that ears, interdigital areas and armpits, where the favored areas for their attachment[38]. However it was later demonstrated that adult ticks prefer to attach to head, neck, ears and also to the back of the dog, making difficult to the dog to remove them. On the other hand, immature stages of this tick life cycle attach to lower areas of the dogs body, such as interdigital areas, legs and belly rump, probably because to their more limited mobility [39].

Once attached to the dog, *R. sanguineus* uses its chelicerae to pierce the host skin and then insert its hypostomeinto the host epidermis. During attachment, ticks secrete a cement-like substance, which forms a cone on the surface of epidermis, while propping for blood, capillary and small blood vessels are lacerated, creating a feeding pool from which the tick extracts the blood [40].

The ticks saliva is a crucial tool to allow the *R. sanguineus* successfully attach and collect its blood meal, once the saliva components suppress the host immune and inflammatory response, allowing the tick to remain on the host for an extended period of time [8].

R. sanguineus reaches sexual maturity and mates solely on the host, the female would not become fully engorged unless mated. During mating, males climbs onto the dorsum of the female and crawls to her ventral surface, and then transfers the spermatophore (a double-

walked, sperm bag filled) to the females genital aperture with the help of his mouthparts, which penetrates the genital aperture [36].

While larvae and nymphs need blood for their molting process, both adult males and females need blood for reproductive purposes, especially females that require large amounts of blood to produce eggs. Whereas males try to mate with as many females as possible, performing small feeds, then transfer a bag of sperm to the opposite sex and die, females mate only once [11].

The drop-off from the host occurs during the day-time for larvae, and during night for engorged nymphs and females. This difference is related to the activity of the host but, also suggests that different strategies are adopted by the tick's different phases of its life cycle [41].

Usually, *R. sanguineus*, life cycle occurs as it follows: an adult female of the *R. sanguineus* species feeds for 5 to 21 days, when the engorgement is complete it detaches itself from the host to digest their blood meal and lay her eggs in a sheltered place. Oviposition is preceded by a pre-oviposition period, ranging from 3 to 14 days. The average duration of the oviposition period is 16-18 days. The females of the species *R. sanguineus* usually lay about 1500 to 4000 eggs (Fig 8) and, after finishing this process, the female dies. The eggs incubation period ranges from 6 to 23 days, after which small larvae hatch from the eggs, staying inactive for 2 weeks. During this period the formation of the external walls of the body takes place and, immediately after this process, the larva starts searching for a host. The larva feeds for a period of 3 to 10 days, before leaving the host to become nymph. The molting period is preceded by a seclusion period, and it may last 5 to 15 days, regulated by molting hormones. The nymph feeds for 3 to 11 days before releasing the host to become an adult, a process that lasts between 9-47 days. The life stages of *R. sanguineus* are present in figure 9 [8, 11, 27, 37].

Fig. 8: Oviposition of *R. sanguineus*: Several engorged females laying eggs, a key process in proliferation [35].

The feeding and molting periods in *R. sanguineus* species are directly influenced by biotic factors, such as host availability and abiotic factors, such as light cycles, humidity and temperature [42]. *R. sanguineus* generally completes two generations per year, but under favorable conditions, the life cycle can be completed in 63-91 days and make up to 4 generations [43].

Fig. 9: Life stages of *R. sanguineus*: Clockwise from top left; larvae, male, female and nymph [37].

When comparing the life cycle of *R. sanguineus* with the one presented by *R. turanicus* it is possible to note several differences namely, *R. sanguineus* is very tolerant from the ecological point of view and as result it is very flexible to a large spectrum of climate conditions. In some areas it is active all year and it has shorter molting periods, so this species has a life cycle with both moderate reproductive success and moderate inter-stage compensatory growth when compared to *R. turanicus*. Although widely distributed *R. turanicus* is ecologically more

limited, what results in a shortest period of activity, higher reproductive rates and faster development including a greater compensatory growth and a higher rate of metabolism [12].

1.2.3 Habitat

A tick's habitat is composed of the variety of living and non-living things in the space in which it lives. Ticks are adapted to two contrasting components of their habitat: the physical environment and their host (fig 10). When ticks are moulting and then questing in the physical habitat they are in danger of drying out and starving. The larvae are most susceptible to predators, such as rodents, birds, reptiles and ants, and also to pathogens, such as fungi. These adverse factors impose some limits to the type of habitats, where a species might be found. However the most important component of the physical habitat of a tick is the climate that is defined by temperature and humidity [36].

R. sanguineus species is the tick with the widest geographical distribution worldwide, currently is established in North, Central and South America, Europe, Africa, Asia and Oceania [3]. As already mentioned this broad distribution is possible due to a number of factors, including globalization, which has increased the mobility of pets, in particular that has occurred since the fifteenth century, which allowed this species expansion, it is believed from the African continent. These factor combined with the ability of ticks successfuly find and establish on new geographic and climatic conditions, with increases in populations of host species and with the great ability to parasitize a very wide host range, beyond the dog, such as migratory birds that can transport them to other habitats and continents, can justify the extension of the worldwide distribution of *R. sanguineus* [4].

Fig. 10: Habitat of *R. sanguineus:* *R. sanguineus* engorged nymphs in a dog kennel, Ivory Coast, West Africa [11].

Another fact that justifies that phenomenon is that *R. sanguineus* is very tolerant from the ecological point of view and also very flexible to a large spectrum of climate conditions [12]. In this regard it was demonstrated that *R. sanguineus* can develop well under different conditions of temperature (20-35ºC) and relative humidity (35-95%) [44].

This tick can survive in very different ecological niches and is particularly well adapted to dry environments. In particular it appears that the species *R. sanguineus* is quite effective at suppressing the rate of dehydration. Furthermore, stages of their life cycle, except the eggs, all can reset the amount of water by absorption of water vapor from the air and by drinking free water. Its ability to retain water, combined with the use of shelters, like vegetation, in order to obtain protection against adverse environmental conditions, allows these parasites to colonize a great variety of habitats. It also appears that this tick is better suited for situations of drought stress than for situations of excess moisture, which is why the *species R. sanguineus* prefers relatively dry environments [45].

It is believed that this species was originally primarily a parasite of burrowing carnivorous like the fox. However, after the domestication of the dog, this animal has become its preferred host as a result this parasite adopted its habitat and therefore is perfectly adapted to live in or close to human dwellings. In high infested domiciles this tick can be found crawling on carpets, wall and furniture [46, 47] .

Therefore, this is an endophilic species, being adapted to life inside dwellings, and is frequent in burrows, artificial shelters or vegetation that lies close to dwellings. However, when in

moist habitats this tick can adapt and adopt an exophilic behavior conducting ambushes to their hosts from the open vegetation. This capacity of changing strategies according to its survival necessities, and the surrounding environments is another indicator that this species is extremely well adapted to survive, perpetuate and colonize different habitats in diverse geographical locations [11].

1.2.4 Population Growth and Abundance

The prevalence and mean intensity of *R. sanguineus* infection in dogs can vary widely both geographically and seasonally. Despite that there are others ecological parameters such as dog population intensity, proportion of dogs treated with ectoparasiticides or tick repellents within the hosts populations, for instance, in areas associated with untreated dogs, the frequency of infected dogs reached 80%, By the opposite in areas where those were adequate treated with repellents, the frequency of infection varied from 3% to 40%. It was also registered that the highest values were associated with dogs that lived in houses with high grassy yards [35].

It has been observed that the extent of parasitic infection in dogs varies within the kennels, indicating that the susceptibility of the individual host is likely to be a significant factor in determining the size of the population of the *R. sanguineus* species, for example it was demonstrated that some dog breed are more susceptible than other, namely the English Cocker Spaniel shows particular susceptibility to be infested by ticks [48].

The resistance of dogs normally, also varies with age, once it was verified that young dogs tend to carry infections with larger number of specimens than older dogs. This fact suggests that there are probably several immune mechanisms involved in limiting feeding and reproductive success of ticks in adult hosts [49]. Besides that, with the exposure, it was noted that ticks that infect naive dogs did not encountered great difficulties to complete its life cycle, in turn, the ones that infested dogs previously infected with ticks produced fewer eggs and weren't able to engorge completely [48].

The dog gender may also affect its resistance to tick infections since it was found that males tend to have a higher prevalence of infection than females, however it is uncertain if this difference is gender related or the consequence of different levels of previous exposure [43]. It was also observed that urban and suburban dogs have a higher prevalence of infection,

particularly dogs that are not systematically treated with ectoparasiticides [49]. Simultaneously, it appears that dogs from rural areas have lower prevalence of infection, the main reason for this phenomenon is probably the low density of dogs in rural areas [50].

However, in spite of these limitations associated with age, previous exposure, gender and race of the dog and even the treatment with ectoparasiticides, in the presence of suitable climatic conditions, easy contact between ticks and hosts, reduction of mortality rates occurs with the increase of the reproductive success, allowing the populations of *R. sanguineus* to growth very rapidly in short periods of time [11].

1.2.5 Seasonality

The weather affects the survival of ticks, especially during the non-parasitic phase of its life cycle, which represents a large part of the lifecycle of this parasite. It also appears that warmer conditions favor its presence and survival, whereas colder conditions hamper their survival. This abiotic factor is extremely relevant to the life cycle of *R. sanguineus,* once unfavorable temperatures, below 14ºC or higher than 35ºC are responsible for causing severe limitations in the development of this parasite [11].

In regions such as the tropics, seasonality is not as evident and *R. sanguineus* species shows no distinct seasonal activity. It was also reported that this parasite is less endophilic, in warmer areas, such as the tropics (Fig.11). In turn, in areas where seasonality is evident, such as Southern Europe, these ticks tends to show reduced activity in winter, that will gradually raise as the temperature increases, reaching the peak of its activity in spring and early summer. Adictionally in some areas there may be a resurgence of activity in the autumn [35].

The peak of activities of this species varies accordingly to its geographic location.In the USA, it occurs in July and September; in France, the spring is associated with a peak of immature stages and the summer with adults; in Greece, the peak of activity of the adult stages occurs continuously in the spring and summer [8]; in Portugal, ticks are particularly active in the months of Abril, May, June, July and August [51].

Fig. 11: Seasonality of *R. sanguineus:* *R. sanguineus* engorged females' crawling in between rocks [35].

Although there are no thorough studies of the role of diapause as a regulatory mechanism, it is believed that diapauses may regulate the seasonality of ticks. It was demonstrated that the light cycles and temperatures that developing ticks are exposed to can affect their feeding behavior [52].

However, *R. sanguineus* species has the ability to present an endophilic behavior when associated with pets, what allows this tick to live in protected locations, thus reducing their exposure to climate change, which leads to a reduction of the effects of seasonality in its lifecycle [37].

1.2.6 Host specificity

Dogs are the primary host of *R. sanguineus* and their presence is a necessary condition for the maintenance of large ticks' populations (fig. 12). However in certain areas *R. sanguineus* displays opportunistically host selection, according to its development stage, once immature stages are often found on rodents and other small mammals and adults usually parasite larger animals [53] .

Fig. 12: Tick infection: A female adult dog highly parasitized by *R. sanguineus* [46].

Despite the fact that the dog is in any circumstance the primary host, there are records of infection by *R. sanguineus* in rabbits, cats, rodents, wild canids, birds and humans [3].

The preference for the host appears to be based on instinct behavior preferences, as a result of the close relation with the dog during the curse of its evolutionary history; however other factors come into play during feeding including modulation of the host immune response. The fact that *R. sanguineus* can occasionally fed on other host namely humans, which normally do not belong to its natural trophic chain, indicates that this tick is able to adopt different strategies to ensure its survival [35].

Although the parasitism in humans is rare, there has being an increase of registered cases over the lasts years, and it's an occurrence that is more common than what was usually recognized [46, 47]. The parasitism in humans is frequently a consequence of an explosive growth of ticks' populations that leads to high level of host exposure [8].

There are a serious of risk factors that increase, the risk of tick parasitism in humans, namely, it was being shown that ticks "attack" humans more frequently when subjected to high temperatures, therefore areas with warmer and longer summer are more dangerous in terms of the risk of the transmission of pathogens [54]. There are also other non-ecological risk factors associated with human parasitism namely, dog ownership, presence of infected dogs indoors

and high level of environmental infections [46]. The increasing number of dogs in many countries is most likely to create conditions more susceptible to increase the risk for humans than what used to occur several decades ago [5].

It was demonstrated that *R. sanguineus* is one of the ticks more capable of pre-feeding on dogs and then move to people, potentially increasing the risk of infection with tick-borne diseases agents [55]. This is one major health concern once tick borne diseases are believed to be responsible for more than 100000 cases of illness in humans' around the world [56].

It was also showed that human parasitism is frequent, but there is no statistically acceptable difference between genders, or different age groups. However rural farmers are associated with the double of the chances of being parasitized by ticks [57]. Another's groups that are considered to be at risk are people who daily contact with dogs, namely veterinarian, pet shop workers and dog owners [8]. Dog owners in particular are associated with 5 times more risk [5].

When a dog bring an infected tick home, the direct risk of pathogen transmission to humans, is minimal, once attached to a dog, ticks will hardly ever detach and move to another host. On the other hand, the introduction of infected larvae and nymphs into a house could result in human infections and tick borne pathogen transmission by the next developmental stages. Moreover the introduction of engorged females may cause the establishment of an in-house population of *R. sanguineus* [58].

1.3 Impact on society

1.3.1 Disease vector role

Since ticks are blood sucking arthropods, they may transmit pathogens, including virus, bacteria, protozoa, helminthes and fungi [35, 56]. They are considered to be the second most important vectors of human disease worldwide, after the mosquitoes, being responsible for 100000 cases of illness in humans throughout the world and are the most important vector of disease-causing pathogens in domestic and wild animals [8, 56].

The infection of a tick by an infection agent occurs during feeding on the host. The mode of pathogen transmission in tick population happens by a transstadial transmission, meaning the passage to the next life stage and by a transovarial transmission, when pathogens are pass on to offspring.[59]. The transmission of pathogens from the tick to its host occurs during the blood feeding (fig. 13). During this process ticks inject saliva, where the great majority of infective forms of pathogens are located, into the skin of the host; a salivary component has the ability to make the tick bite initially painless allowing it to go undetected for relatively long periods of time. This mechanism allows this parasite to feed more easily but, simultaneously, increases its effectiveness as a pathogenic vector. It is also verified that a higher dose of this anesthetic component is associated with a higher probability of transmission of pathogens [8].

Fig. 13: Tick infection on humans: (a) *Hyalomma marginatum* male feeding on a man in southern Italy. **(b)**. Skin reactions 24 hours after being bitten [58].

R. sanguineus tick constitutes a possible risk to public health, because it can be responsible for the transmission of a wide variety of pathogens to dogs and humans. The most troubling

canine illnesses transmitted by this tick are babesiosis, caused by *Babesia canis* and monocytic ehrlichiosis, caused by *Ehrlichia canis*. The most dangerous pathogens transmitted to humans are *Rickettsia conorii*, which is responsible for causing Mediterranean spotted fever and *Ri. rickettsii*, the etiologic agent of Rocky Mountain spotted fever. All of these diseases are associated with morbidity or even death if not treated properly [4, 8, 11, 58].

In severe cases, babesiosis is responsible for causing haemolytic anemia, hypotensive shock, intravascular coagulation, systemic inflammatory response and multiple organ dysfunctions, erythrocyte autoaglutamination and oxidative damage in red blood cells. The susceptibility to this disease varies with the host, its breed and its age, and also the auto-immne status of the dog. In severe cases, Erlichiosis causes immunological destruction of platelets, platelet dysfunction, ocular and central nervous system abnormalities and, in some cases, bone marrow destruction. Certain dogs breeds and younger dogs are more susceptible to this disease [4].

Rocky Mountain spotted fever and Mediterranean spotted fever are characterized for presenting one weak incubation period, frequently asymptomatic, followed by the arise of symptoms such like, headache, myalgia, arthralgia vomits, diarrhea, physical pain, and after one weak, skin spots spots with 0,5 to 2 cm of diameter appear, where the tick has bitten [59]. Severe cases are associated with very high fevers, lethargy, anorexia, icterus, hyperthermia, and trombocyptoria [60].

R. sanguineus species is also associated with the transmission of other pathogenic agents, such as *Anaplasma marginale*, *A. platys*, *B. cabalii, B. canis canis*, *B. canis vogeli*, *B. gibsoni*, *Coxiella burnetii*, *Dipetalonema dracunculoides*, *Mycoplasma haemocanis*, *Rangelia*, *Salmonella spp*, among others [11, 58, 61].

In addition to the entire pathogens previously mentioned, the filaroid *Cercopithifilaria grassi* is associated with dermal microfilariae, is also transmitted by *R. sanguineus.* This was demonstrated by the fact that this filaroid appears in every stage of the life cycle of this tick that also presents a high level of toleration to its infections [62].

There is also the suspicion that this parasite can transmit the bacteria *Leishmania infantum*, responsible for causing visceral leishmaniasis that can affect both dogs and children. This

suspicion gained further relevance after a study that detected and quantified DNA of *Leishmania infantum* in field collect engorged females, eggs and larvae, providing further evidence of the transovarial passage of these bacteria in *R. sanguineus,* what support the hypothesis of this tick as effective vector of *Leishmania infantum* [42].

R. turanicus species is also associated with the transmission of pathogens to humans in particular *Ri. conorii* and *Ri. massiliae*. Nevertheless it appears that this species has a much smaller amount of associated pathogens, as a result of this species feeding less frequently in humans and/or being misidentified. So there is need to deepen the existing knowledge on which are the transmitted pathogens, as well as clarify which ones are transmitted by each species of ticks [11].

R. sanguineus can act as vectors and as reservoirs of certain pathogens, once it has the ability of maintaining the pathogen in nature, through several generations by transvorarial and transtadial transmission; dogs can also be a reservoir host, once they can be infected by certain diseases without showing any symptoms [11]. Ticks can be naturally infected by microorganisms like bacteria, and trypanosomatrids of unknown pathogenicity. The prevalence of *R. sanguineus* infections by the pathogens it carries may vary greatly from region to region [8].

Little is known about the interactions of ticks and its pathogens; it was showed that bacterial community of *R. turanicus* is highly dominated by *Coxiella* and *Rickettsia*, both associated with low taxonomic diversity. It also appears that the density of *Coxiella* is higher in females. In turn, *R. sanguineus* presents *Coxiella* in the egg, larvae and adult stages but always associated with the prevalence of higher intensities of *Rickettsia*. The densities of both bacteria were similar in both genders, but presents seasonal variation. These results point to an obligatory and facultative association between the 2 tick species and *Coxiella* and *Rickettsia sp* [63].

The theory that the interactions between *R. sanguineus* and several pathogenic agents is far from being the perfect symbioses has gained support after histological analyses revealed that the infection with *R. rickettsii*, can interfere negativity with the reproduction of ticks [64]. It was showed that ticks infected by *R. conorrii* evidenced lower average weight in engorged females and eggs, and also that infected nymphs, that were exposed to low or high

temperature for a month, experienced higher mortality when transfer to 25ºC, than non infect ticks. The same was observed in adults, suggesting that infected ticks may not survive the winter [65, 66].

Tick borne diseases are increasing worldwide and it is believed that the numbers are under estimated; in association with this problem its diagnostic can be challenging [67, 68]. The expansion of three host ticks trough the world shows, that this type of tick evidence a great potential for colonizing new habitats. This capacity constitutes a threat to public health once human activity that promotes the fragmentation of habitats has been increasing in the last decades, and such behavior is associated with higher risk of exposure to tick borne diseases. Simultaneously, climatic changes will provide new opportunities for the expansion of ticks population, into uninhabited regions, in addition domestic animals are reservoirs of tick borne diseases and act as host bridges in emerging and re-emerging pathogens to humans [69].

For such reason tick control must constitute a priority to improve human and animal health worldwide [56]. Maintaining pets on effective control using adequate products, such as repellents and ectoparasictides, and the awareness campagns of dog owners and populations are fundamental for this issue, which will constitute effective measures against this threat to public health [58].

1.3.2 Control

Considering the threat that tick borne diseases represent to public health, it is possible, to claim that the control must be a priority to improve human and animal health worldwide [56]. Maintaining pets on effective control, using adequate products such as repellents and ectoparasictides in order to prevent the initial attachment and also the avoidance of tick-infected areas, the reduction of tick habitat close to human homes are effective measures to achieve this goal [55].

Dogs can be treated with a diverse sort of veterinary preparations, such as formulations impregnated collars, sprays, shampoos and powders, fipronil, amitraz, carbaryl, and pyrethroids (detamethrin, permethrin, and cypermethrin) are among the most frequently used acaricides for controlling *R. sanguineus* ticks [70].

Despite that, it is important to consider that when it comes to tick control, only 5% of ticks are on the dog, the remaining 95% are on the environment, therefore the effective elimination of tick population will require an integrated control strategy targeting the canine population as well as the environment. However the environmental treatment can only be effective, when restricted areas are concerned and such success depends on a number of factors, such as level of environmental infestations, presence of infestation in areas next to the treated area, residual effects of the acaricides and environmental conditions [8].

Unfortunately the long term use and the misuse of acaricides is a serious problem that may result in environmental pollution, and acaricide resistant in ticks [28]. Several studies suggest that ticks are highly resistance against pyrethoids acaricides, and also that the resistance against a certain acaricide may vary according to the region [8]. In the acaricide department, one substance that reveals great potential is the fluaziron, once it was demonstrated the susceptibility of *R. sanguineus* nymphs to various concentration of this compound.It inhibits the synthesis and deposition of chitin in the target organism preventing the moulting of the ectoparasite to the next stage. This compound is an upgrade on its predecessors being more effective than most of them and it is also more specific, so it does not induce resistance on target organisms and decreases the risk of environmental contamination [71].

It is suggested to use, non-chemical control in conjunction with chemical control; in terms of habitat chances the sealing of cracks and crevices, and keeping the grass short are highly recommended measures [8]. Simultaneously, the veterinarians also play an important role in this subject, once they are responsible for educating dog owners to the severity of this issue, and instruct them to examine, locate and remove the ticks from their dogs periodically [58].

It is also believed that non-chemical control may be the future. Ticks have many natural enemies namely bacteria, fungi, nematodes, and larger predators, but few species have being evaluated as tick biocontrol. Some laboratory results show that several bacteria are pathogenic to ticks, but their mode of action and their potential as biocontrol agent remains to be determinate. The most promising entomophatogenic fungi appears to be *Metarhizium anisopliae* and *Beauveria bassionis*, there is also potential in wasps of the genus *Ixophages* [72].

Biological control is likely to play a considerable role in future programs for tick management, namely because the methods are far more specific in their selection of target pest than acaricides, and ecological and environmental problems are minimized. However

there are some obstacles to overcome, namely the education of the consumers and a slower effect when compared to the alternative chemicals [72].

Another interesting solution would be the creation of a competent anti-tick vaccine, but currently there is no such immunotherapy available. Despite the identification of protein codified by genes from tick saliva, it might be helpful to the discovery of potential targets for anti-ticks vaccines; dogs appears to develop immunity against ticks and this is an important limiting factor for the development of a vaccine, as result the progress to achieve such goal has been low [8].

1.3.3 Economic Impact

Even without acting as a vector of disease, ticks can be harmful to livestock and of great economic importance simply to their direct effects, depending on circumstances namely: the ticks' species involved, the susceptibility of the livestock in the region and especially the climatic conditions. Uncontrolled tick infestation in climatic favorable conditions seriously affects European cattle to an extent that a choice has to be made between simply renouncing the use of such cattle or applying intensive and expensive chemical tick control, which usually leads to a rapidly increase tick resistance against the used acaricides. The damaged caused by ticks bites also diminishes the value of skins and hides for the manufacturer of leather, certain ticks may cause the loss of teats or lameness, depending on the site of attachment, which leads to the increase of calf mortality; and some ticks species contain paralyzing toxins, that can cause death even in adult cattle [28].

When focusing on the diseases transmitted by ticks to domestic ruminants, the most important are babesiosis, theiliriosis, anaplasmosis and cowdriosis however recovered animals retain the infection and remain immune for long periods. Besides in areas where such diseases are endemic, the local livestock has been exposed to a long process of natural selection and become more tolerant. Despite that fact, the global cost of diseases transmitted by ticks and their control has, been estimated at 7 milliard US dollars. Although an outrageous number, it actually seems legit, when taking into account, for instance thatthe annual loss dues to cowdriosis in Zimbabwe, reaches 6 million US dollars; the annual loss associated to theileriosis in India reaches 384,3 million US dollars, and in eastern, central and southern Africa it reaches 168 million US dollars [28].

Companion animals, in particular dogs, also pays a heavy tool due to tick borne diseases, several babesiosis are highly pathogenic to dogs. The same occurs with erlichiosis and infections by *Erlichia canis* are often fatal. Dogs belonging to tourist travelling to warm regions constitute a risk group in terms of the susceptibility to contract such diseases. For such motives, from the viewpoint of the animal health industry, acaricides for companion animals are an increasing market focus. Simultaneously diseases such as *Babesia cabally* and *Theileria equi* are known to have a great economic impact on the horse industry [28].

1.4 Genetic studies

1.4.1 Molecular identification of species

Eukaryotic organisms also have DNA in mitochondria (mtDNA), which is separated and distinct from the nuclear genome [73]. In animals it occurs as a single double helical circular molecule containing 13 protein-coding genes, 2 ribosomal genes, a non-protein and several transferences RNA's [74]. Also contains a large non-coding region know as control region D-loop that is the main responsible for the differences in size of mtDNA between species [75].

It is believed that, in animals, such distinctions turn mtDNA more suitable to be use as a molecular marker than nuclear genome. There are several factors that contribute for that fact, namely mtDNA is easy to isolate, once it presents high copy number, lack of introns, limited exposure to recombination, high mutation rates in different regions of the molecule; [76] and its mode of inheritance once it's maternally inhered through cytoplasm [73]. Simultaneously, eukaryotic cells contain hundreds to thousands of mitochondria and each one contains several copy of mtDNA [74].

It is suposed that molecular genetic studies in arthropods vector would fill gaps in the understating of this type of vector [77]. One key factor for the success of such studies is the selection of an adequate marker, in that moment all competent markers must be weighted, and decide upon the one who's more suitable for the purpose in question [78].

It was concluded that the ideal marker should possess the following properties: a single copy gene may be more useful than multiple copy genes; as marker genes sequences are aligned prior to phylogenetic analysis, their alignment should be easy; the substitution rate should be optimum as to provide enough informative sites, a gene evolving too fast may reach a state of

saturation due to multiple substitutions; Primers should be available to selectively amplify the marker gene, however the primer should not be too universal as in that case it would lead to amplification of non-specific genes present as contaminants; too much base variation among the taxa, is not preferable which may not reflect the true ancestry [79, 80].

The fact that genes 16S and 12S both produce fragments with less than 450 bp, making it easier to obtain in a single reaction and both present slow rates of evolution, suggesting that they might be adequate for being used as an molecular markers and a tool to reconstruct phylogenies [81].

The 39S and 28S ribosomal subunits contain respectively the 16S and 12S RNA species encoded by the mtDNA. It is believed that the 16S gene has a structural role being involved in several biological processes including protein syntheses, namely acts as scaffold defining the position of ribosomal proteins. It is involved in binding the two ribosomal subunits, and in stabilizing the correct codon-anticodom pairing by the formation of hydrogen bonds. It is suposed that mutations of this gene lead to ribosomes with deficient functionality in prokaryotes this gene contains the shine-delgado sequence in the 3´ end. On the other hand, 12S gene also has structural role in several biological processes, namely a second putative initiation site for H-Strand-transcription located around the nucleotide 638. Mutations on this gene are associated with hearing loss in humans [82].

1.4.2 Molecular Markers associated with *R. sanguineus*

Due to the fact that ticks belonging to the genus *Rhipicephalus* are extremely difficult to identify morphologically, molecular methods are becoming, increasingly important in systematic acarology [80].

There are several nuclear markers that had been used frequently in this areas namely 18S Deoxyribonucleic acid (DNA) and 28S DNA, also ribosomal markers, such as internal transcribed spacer of the nuclear ribosomal gene cluster (ITS1 and ITS2), mitochondrial ribosomal genes, like 16S and 12S, have also been utilized with that context and, of course, the mitochondrial marker cytochrome oxidase I (COI or Cox 1), cytochrome oxidase III (COIII), and Cytochrome B (CYTB) [77].

It was demonstrated that ITS2 and COI together provide a powerful tool for studies of intraspecific variation and phylogenies of closely related species; 18S DNA and 28S DNA are equally useful for phylogenetics at the other end of the taxonomic spectrum. In turn, the markers 12S and 16S might be useful for use at intermediate taxonomic levels between genus and family [80].

A comparative analysis of the nuclear and mitochondrial markers was performed namely using the markers 16S, 12S, Cox 1 and ITS2. It was confirmed that all these markers had the capacity to differentiate the Rhipicephalinea species examined. Molecular identification was also supported by the distinct separation of species-specific clades inferred from the phylogenetic analysis of all mitochondrial sequences, however little interspecific divergence was detected amongst ribosomal ITS2 sequences, of the *R. sanguineus* complex, which resulted in the ambiguous placement of certain sequences in the corresponding phylogenetic tree. Despite that it was confirmed that the mitochondrial deoxyribonucleic acid (mtDNA) markers are suitable and reliable for the identification of ticks within the *Rhipicephalus* genus. In fact, these markers constitute a powerful tool for future studies of taxonomy, speciation and evolution of this group of ticks [78].

A similar study was performed using the markers, 16S, 12S, COI and ITS2, concluding that ITS2 is more adequate for species of the genus *Ixodes*, than for species of the genus *Rhipicephalus* and 16S is the one with the highest primer efficiency. Simultaneously, it was also observed that 16S and 12S produce fragments with less than 450 bp, making it easier to obtain in a single reaction, and that the primer 16S is the sequence with highest sequence quality, followed by COI, despite that 5`region is standard marker for DNA barcoding. This originates the deposition of a large number of COI sequences from animals in databases, such as Barcode of Life Data Systems (BOLD) and GenBank. For that reason, COI might be considered a first choice, however it has proved to be of limited use in identification of some species and in the case of its failure, 16S, 12S, and ITS2, are all competent alternatives specially 16S [81].

1.4.3 Population genetics

Ticks from the *R. sanguineus* group are historically associated with one of the groups, around which there is less consensus. Ticks belonging to the genus *Rhipicephalus* are extremely

difficult to identify morphologically, due to the high level of intraspecific variability. To promote more consistent taxonomic reconstructions, morphological studies should be applied together with biological and molecular studies. The mitochondrial 16S and 12S (rDNA) ribosomal DNA target regions have been the most sellected for such purposes [8].

In 1994 the mitochondrial marker 16S was used to infer the phylogeny of hard and soft ticks, what allowed to demonstrate that his marker was adequate to perform genetic studies in ticks but also, that in geo-chronological terms the origin of Ixodidae, occurred somewhere in the late Cretaceous. It was also demonstrated that *R. sanguineus* and *R. turanicus*, presented some considerable genetic differences [19]. Posteriorly, these results were confirmed and it was concluded that this marker was quite suitable for species of ticks closely related but also useful for comparison of distant related taxa [21]. These findings were supported and extended to the molecular marker 12S, by several authors [83]. The marker 12S was also used in conjunction with COI and ITS2 to infer the phylogeny of ticks. These studies provided strong evidence that the *Rhipicephalus* species were paraphyletic with respect of the species of the genus *Boophilus* [20, 22].

Using the 12S mitochondrial marker, combined with the analysis of morphologic features it was concluded that the sequences of *R. sanguineus* from the northwestern Mediterranean shore and the sequences of *R. turanicus* from Turkmenistan, represent one single species; in turn when the *R. sanguineus* Mediterranean sequences were compared with the *R. turanicus* sequences isolated in South African specimens, the genetic difference increased considerably. A similar situation occurs when comparing French sequences with sequences isolated in Zimbabwe taking these observations into account it was suggested that in the case of ticks 12S gene at least divergence up to 7,8% indicates an intra-specific variation, and only higher values suggest the presence of inter-specific variation [18].

These molecular markers, not only allow to study, how different species are genetically related, but also how that relation occurs in different populations. Until recently it was believed that *R. sanguineus sensu stricto* was the only representative of the genus in South America [3]. However the fact that relevant morphologic differences were found between *R. sanguineus*, from Brazil and *R. sanguineus* from Argentina, namely it was observed that the females from Brazil presented a genital aperture V-shape, a typical characteristic of *R. turanicus*, on the other hand females from Argentina displayed U-shaped genital aperture a

typical characteristic of *R. sanguineus* suggested the existence of at least two different populations, in South America [84].

As consequences of these findings, study using the molecular marker 12S demonstrated that the populations of Rafaela, Santa Fé, Argentina and the population in Jaboticabal S. Paulo, Brazil revealed considerable genetic differences. It was also possible to conclude that populations of *R. sanguineus* in Argentina were closely related to the European populations of *R. sanguineus* and on the other hand, populations of *R. sanguineus* of Brazil were closely related to the African populations. These conclusion are supported by crosses between the Brazilian strains and Argentine strains of *R. sanguineus* from which some hybrid larvae were obtained but the adults were infertile [85].

In this context, the comparison between genetics strains from Brazilian ticks with origin from several regions of that country was performed using the mitochondrial molecular markers 12S and 16S. Considering the results, relevant genetics differences were found among the several Brazilian sequences and overall strong genetic relation were detected between *R. sanguineus* from Brazil and Asia (Taiwan and Thailand) and also with *R. turanicus* from Africa (Zimbabwe and Zambia). On the other hand, populations of *R. sanguineus* from Argentina and Uruguay appeared to be related to French, Egyptian and North American sequences [86].

A similar result was obtained when using the 16S molecular marker with the purpose of comparing genetic sequences from several European and South American Countries. Results displayed the formation of two clades, one formed with sequences from *R. sanguineus* and *R. turanicus* with origin in Mexico, Costa Rica, Panama, Colombia, Venezuela and South Africa and another one formed by sequences from *R. sanguineus* and *R. turanicus*, with origin in Europe and also Chile, Argentina and Uruguay. These differences between the two clades suggests the existence of two species, one associated with tropical climate, and another associated with temperate climate, and lower temperatures [87].

Posteriorly, an analysis of *R. sanguineus sensu lato*, using the molecular markers 12S and 16S, was performed in the Southern cone of South America. It was verified that when, phylogenetic analysis was effectuated, the formation of two groups occurred, representing the southern lineage and the northern lineage. The southern lineage is formed by haplotypes, isolated in Argentina, Uruguay, Chile and Italy. This lineage is closely related to European sequences and is associated with temperate areas. The Northern lineage is constituted by sequences isolated in Mozambique, Brazil, Paraguay, Colombia, S. Africa, and in the North of

Argentina. This lineage is closely related to African species, and is associate with tropical climate [88].

A similar study was conducted in North America using the molecular marker 12S. It was observed that sequences from Oklahoma were closely related to sequences isolated in Israel and they were significantly distant from the ones isolated in South Africa. It was also demonstrated that the phylogenic analysis inferred with this gene revealed, sequences of *R. sanguineus* fromLos Angeles, Atlanta and Arizona are closely related to sequences previously isolated in Rafaela, Argentina, and in turn sequences of *R. sanguineus* with origin in Saint Kitts are closely related to the tropical lineage. These data were supported by crosses simultaneously conducted between North American ticks, Mediterranean and African ticks [10].

Full mitochondrial genome was sequenced for *R. sanguineus* from China and *R. sanguineus* from USA, considering the 13 protein-coding genes, comparison revealed divergences that ranged between 9,34% to 15,65%. In addition, sequence comparison of the gene Cox 1 and CYTB, among *R. sanguineus* revealed substantial nucleotide difference between populations of *R. sanguineus* from China and USA. These findings suggest that both populations are likely to be separated species. What supports the proposal that *R. sanguineus* tick complex may represent a species complex of at least two closely related species [24].

Another phylogenic study using 3 molecular markers; 16S, 12S and Cox, revealed the existence of at least four different phylogenic groups. It was also demonstrated that *Rhipicephalus sanguineus sensu lato* is associated with the northern lineage, and *Rhipicephalus sanguineus* T2 is associated with the southern lineage.In addition to this two previous known lineages, another phylogenic clusters where formed, namely one constituted by the sequences isolated in *R. turanicus*, from Italy, Israel, and Switzerland, and also *Rhipicephalus sanguineus* T1, formed mainly by sequences isolated in Greece, all 4 groups, present morphologic divergences, that support the genetic difference verified [32].

The results of these studies reinforce the hypothesis that at least two species need to be redescribed and delineated especially considering, that they may differ in their ability to transmit pathogens as well as in the frequency of contact with humans and domestic animals [11].

1.5 *R. sanguineus* in Portugal

The climate in Portugal may be considered oceanic along the littoral and Northern islands, and Mediterranean in the South. The annual temperature varies between 16ºC to 26ºC in summer time and 3Cª to 13Cª in winter, such factors constitute favorable climatic conditions for the distribution and maintenance of ticks and tick borne diseases in nature. In addition,there are other ecological conditions that favor the proliferation of ticks in Portugal, namely the occurrence of a large variety of susceptible hosts and adequate vegetation [89, 90].

When focusing on cattle, it is believed that species associated with tick borne disease are spread through the entire country, with higher incidence in Alentejo and Ribajeto. The more relevant diseases transmitted to cattle are Babesiosis and Theileriosis [89].

Currently, there are 21 species of ticks identified in Portugal, and the diseases with the highest impact on public health are lymes disease and boutonneuse fever. This last one is an endemic disease in Portugal mostly caused by *R. conorii*, a pathogen transmitted by *R. sanguineus* considered to be the most abundant species in Portugal. It is believed that maintains its activity during the all year, once it appears to be perfectly adapted to the continental conditions of temperature and humidity [91], with its peak of activity established in the months of July and August [59].

In Portugal, boutonneuse fever is very frequent over 1000 cases are witnesses every year, being one of highest rates in the Mediterranean countries. However these numbers are underappreciated, even considering that this is one disease of mandatory declaration [92]. Usually this disease has a benign resolution, when early diagnostic is made, however severe cases may occur and some of them, unfortunately lead to death. The number of mortal victims in Portugal is also one of the highest when compared to other European endemic countries, being the district of Bragança with the most reported cases. Althought there is no homogeneous distribution between genders but, the most affected age group are children from 1 to 4 years old [91].

In addition to *R. conorii*, the pathogen responsible for boutonneuse fever, the first tick borne disease diagnosed in Portugal, other pathogens were described in Portuguese specimens of *R. sanguineus*, namely *R. massiliae* and the virus Thagoto of unknown pathogenicity. Simultaneously *Coxiela bunetti* was also detected, but still not isolated [59].

Portugal is not indifferent to the main question regarding the complex *R. sanguineus*, the taxonomic issues between *R. sanguineus* and *R. turanicus*, in that context both species had been previously described in Portugal. It was also noted, that *R. sanguineus* appears to be mostly associated to the domestic dog, in turn *R. turanicus* seems to be more related to ruminant cattle, namely sheep [93]. However posteriorly, it was proposed that these two species were genetically indistinguishable [94]. Considering that both species are associated with different pathogens and different levels of risk to public health and also that Portugal has the adequate geo-climatic conditions for the proliferation of ticks and ticks borne diseases it seems important to clarify this issue.

2. Dissertation context and aims

The Portuguese populations of *R. sanguineus* have been previously studied, however, there is still much controversy and few consensus, namely in relation to the correct identification of *R. sanguineus* and *R. turanicus*, once that both species had being formely described in the country, but recent studies indicate that they are genetically similar.

It is in this context that the present study is integrated, consisting in a preliminary morphological and molecular analysis of Portuguese populations of *R. sanguineus*, collected in dogs from the outskirts of Lisbon. This is definitely a relevant question once that this particular species of tick is associated with several tick borne diseases that can be transmitted to both dogs and humans. Furthermore, Portugal is one of the European countries with one of highest rates of incidence of tick borne diseases, namely boutonneuse fever. Therefore the objectives of this study are:

- Analyzing the morphological variability in the Portuguese populations of *R. sanguineus.*
- Confirm if the morphological variability is accompanied by genetic diversity.
- And also if there is enough genetic variability to justify the classification in more than one species.
- Evaluate which qualitative and quantitative variables are more suitable to distinguish *R. sanguineus* from *R. turanicus.*
- Investigate if the molecular markers 12S and 16S are adequate for this type of molecular analysis.
- Compare the sequences obtained in Portuguese populations of *R. sanguineus* with other worldwide and understand how they are phylogenetically related.

3. Materials and Methods

3.1 Tick collection and identification

The ticks that compose the used sample in this study are all part of the collection of the Instituto Superior de Agronomia (ISA) (formely as a part of the Collection of Zoology of the PortugueseTropical Research Institute, CZ/IICT). They were all retrieved from dog hosts, during a time period of 9 years, from 2005 to 2015, in the geographic areas of Setúbal, Alcobaça, Alcochete, Peniche and Vila Franca de Xira. The species identity, of each specimen, was determined through the resort of keys and descriptions, namely *R. pusillus* [95] *R. sanguineus* [27, 32, 95] and *R. turanicus* [27, 32, 95]. The intermediate forms were classified according to Dantas-Torres 2013, mostly as *R. sanguineus* Type 1 and *R. sanguineus* Type 2, however some ticks have presented morphological characteristics different from the species described so far, and so, were classified as *R. sanguineus* and *R. sanguineus* D in the case of males, and *R. sanguineus* Intermediate in the case of females.

3.2 Morphological and Statistical data analysis

A total of 475 ticks (239 males and 236 females) were included in this study. Specimens that were engorge, sub-adult stages or poorly preserved and damaged, were excluded from this analysis, once all these factors would increase the difficulty of the challenging task of identify specimens. Through observation of its morphological characteristics around 3800 pictures were taken to all the specimens contained in our sample. This was effectuated using a light stereomicroscope, connected to live measurement software Leica Application System (LAS) (Leica Application System 2009). The purpose of this extensive task was to analyze and measure a series of quantitative and qualitative variables for both males and females. The quantitative variables considered for the male sample were: Conscutum length; Conscutum width; After posteromedian grooves measured width; Capituli dorsal basis width; Capituli dorsal basis length, Spiracle areas max length; Spiracle areas max width; Spiracle areas width 1st/2st third; Spiracle areas 2st/3st third; Spiracle areas ending width tail; Spiracle areas ending width adjacent; Spiracle area angle, Adanal plates height and Adanal plates width. The qualitative variables considered were: Conscutum punctuation distribution; Conscutum

punctuation size; Cervical fields depressions, Cervical fields shape Cervical Grooves definition; Cervical setiferous punctuations; Capituli ventral palps height, Palp shape (2^{nd}); Lateral grooves beginning; Lateral grooves festoons ending; Lateral grooves texture; Posteromedian grooves (short or long); Posteromedian grooves deepness; Paramedian grooves shape; Paramedian grooves deepness; Parma; Spiracular areas Type; Adanal posterior margin; Adanal plates total shape and Adanal plates ending. The sexual dimorphism led to the necessity of creating a different set of variables for the female sample, and so, the quantitative variables considered, for females were: Scutum length; Scutum height; Capituli basis width; Capituli basis height; Porose areas height; Porose areas width; Spiracle width; Spiracle height; Spiracle width of the tail mouth; Spiracle ending tail width; Spiracle tail length; Spiracle area angle; Genital pore aperture; Sclerites length; Sclerites width; Sclerites insertion to aperture upwards and Sclerites insertion to aperture downwards. As to the qualitative variables, the ones considered were: Scutum punctuation distribution; Scutum punctuation size; Scutum posterior margin; Cervical fields depressions, Cervical fields shape; Cervical Grooves definition; Cervical setiferous punctuation; Palp shape (2^{nd}) and Genital aperture pattern. Spiracles and adanal plates in the case of males, and Scutums and Genital regions in the case of females were prepared in a slide in order to clarify internal structures, and highlight certain details, morphologic clusters were then formed. Most variables included in the statistical analysis were treated trough a ratio, in order to make these easier to interpreter and to establish relations. All the variables include in this study, were subjected to hierarchical cluster analysis, which was performed separately for quantitative and qualitative variables and separately for both genres due the morphologic dimorphism displayed between males and females. This analysis was conducted in SSPS software [96] using the ward method and the square of the Euclidian distance, Quantitative variables were analyzed using an Analysis of Variance (ANOVA) in conjunction with the Tukey Honestly Significant Difference (HSD) test and the qualitative ones trough a Cross-Tabulation. After that, the qualitative and quantitative clusters formed were compared by resorting to correspondence analysis, however the comparison between the qualitative clusters with the morphologic clusters and the ones between quantitative and morphologic clusters was performed trough a relative frequency analysis, in other to make, certain elations more innate. The purpose of the morphologic analysis in conjunction with the statistical analysis is to observe if the obtained clusters are associated with individuals that share the similar morphologic characteristics, and evaluate if there is an association between the morphologic clusters and the ones obtained

with qualitative and quantitative variables, and simultaneously evaluated, with morphologic features are associated with each cluster.

3.3 Genetic Analysis

After a detailed morphologic study, 146 representative tick specimens were selected for genetic analysis. DNA extraction was performed using a commercial kit E.Z.N.A Insect DNA Kit according to the manufactures instructions [97]. However, DNA was only extracted from ticks' legs, in order to allow the return of the specimens to the original collection, and also because the sequences of DNA acquired, might be integrated in national and internationals data base, and so it is mandatory to keep intact at least half of the specimen, from which the sequence was isolated.

Once the extractions were completed, it was resourced to the spectrophotometer, Nanodrop 2000 UV, Therm Scientific, with the purpose of evaluating the quality and concentration of the acquired DNA.

Posteriorly, the amplification of DNA was effectuated, using the Polymerase Chain Reaction (PCR) method. Each reaction was performed with a total volume of 25 µl , composed by 5 µl of tick genomic DNA and 20 µl of PCR mix, containing 13,15 µl of ddH_2O, 2,5 µl of Buffer, 1,25 of µl of $MgCl_2$, 0,1 µl of Taq (Nzytech), 1 µl of mixed dNTP's and 1 µl of each primer. In all PCR reaction, to avoid contamination problems, negative and positive controls were added to run simultaneously. PCR conditions included an initial denaturation step at 94 ºC for 2 minutes, followed by 35 cycles for 45 s at 94 ºC, 45 s for primers annealing at 55 ºC and 45 s for primer extension at 45 s; a final extension step was carried out for 7 minutes as 72ºC. The primers used for amplification and sequencing were previously described by [19, 85, 86] (table 1). A portion of amplified product were examined by 0,5% agarose-gel electrophoresis, followed by staining with green buffer (3 µl DNA + 2 µl dye), PCR strains were then subject to UV-light, and compared to the ladder. Successful amplifications were purified with the SureClean Plus kit [98] and sent to sequence at Macrogen Europe.

Table 1- Primes used in the amplification of 12S and 16S DNA [19, 85, 86]**.**

	12s rDNA	**16s rDNA**
Forward	AAACTAGGATTAGATACCCTATTATTTTAG	CTGCTCAATGATTTTTTAAATTGCTGTG
Reverse	CTATGTAACGACTTATCTTAATAAAGAGTG	TTACGCTGTTATCCCTAGAG

The sequences were then analyzed and treated with the assistance of informatics programs, such as BioEdit V7.2.5 [99] and DNA Baser Sequence Assembler V4 [100]. Alignments were then analyzed and the haplotypes acquired were compared to other sequences available at GenBank. The pairwise-distance and the absolute number different nucleotides were calculated using Molecular Evolutionary Genetics Analysis (MEGA) software [101].

In order to investigate the phylogenic relation among the sequences isolated in ticks, it was used MEGA software to perform a Neighbor-Joining (NJ) method comprised in the Tamura-Nei model. The phylogenic analysis and the bootstrap values were based on 1000 replicates and partial sequences of 12S rDNA and 16 S rDNA of *Rhipicephalus* spp., available on GenBank were also included.

4. Results

4.1 Statistical and morphologic analysis - Males

4.1.1 Hierachical cluster analysis

Hierarchical Cluster Analysis was effectuated separately for quantitative and qualitative variables; this analysis was conducted in SPSS software [96] using the Ward method and the square of the Euclidian distance. This allowed obtaining the dendograms presented in Fig. 14 and Fig. 15

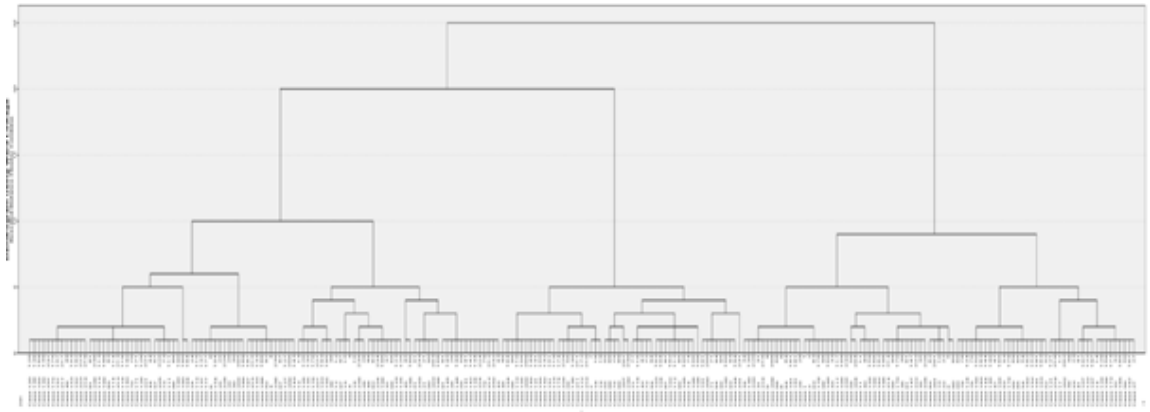


Fig. 14– Hierarchical Cluster Analysis dendrogram obtained with males' quantitative variables data. The higher distance between fusions coefficients were obtained in the rescaled distance value 20 (forming 3 clusters).

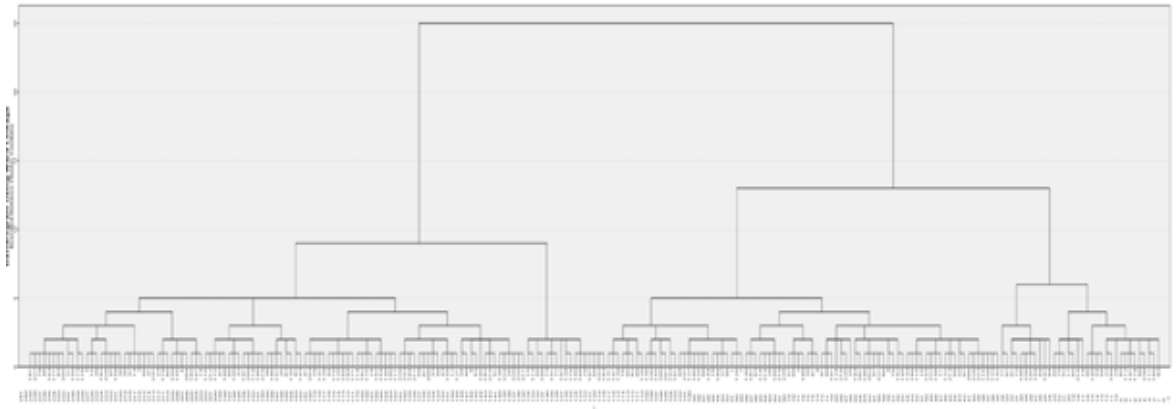


Fig. 15: Hierarchical Cluster Analysis dendrogram obtained with males' qualitative variables data. The higher distance between fusions coefficients were obtained in the rescaled distance value 13 (forming 3 clusters).

Besides these dendograms, the Cluster analysis has also provided fusion coefficients, the subtraction between the last 10 fusion coefficients allowed acquiring the differences between the fusion coefficients; the biggest differences indicate the most appropriated numbers of clusters to be formed. These data are presented in Table 2.

Table 2 - Last 10 fusion coefficients obtained with the Hierarchical Cluster Analysis.

Males					
Quantitative V.			Qualitative V.		
N Clusters to form	Fusion coefficients	Subtraction of fusion coefficients	N Clusters to form	Fusion coefficients	Subtraction of fusion coefficients
2	1904,000	244,146	2	189,219	17,344
3	1659,854	186,238	3	171,875	8,840
4	1473,617	90,035	4	163,034	6,145
5	1383,582	79,610	5	156,089	3,805
6	1303,972	55,121	6	153,085	3,301
7	1248,851	46,745	7	149,784	3,181
8	1202,106	46,214	8	146,603	2,795
9	1155,893	44,412	9	143,808	2,303
10	1111,481	41,294	10	141,506	2,259
11	1070,187	39,280	11	139,246	2,105
-	1030,907	-	-	137,142	-

Note: These values were used to determinate the number of clusters to form in the different statistical analysis performed. N- number, V. - variable.

In order to make these data easier to interpret, both quantitative and qualitative variables were represented using graphics and were presented in graphical form, in figure 16 and 17.

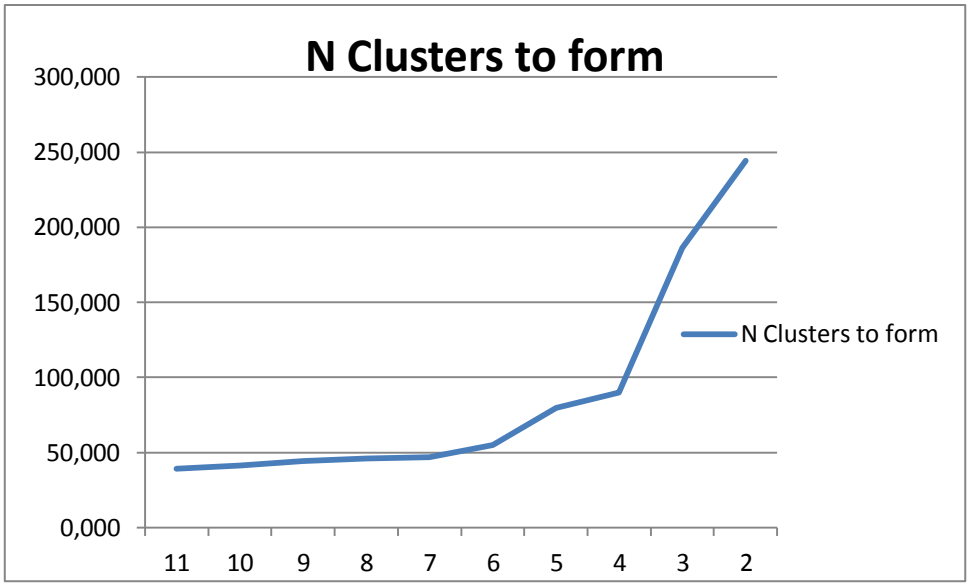


Fig. 16: Quantitative variables- the subtraction between the last 10 fusion coefficients, gaves the differences between fusion coefficients' the biggest differences, indicate the more appropriated number of clusters to form, in this case, the biggest differences between fusion coefficients occurs in the position 3, followed by the position 2, therefore 3 or 2 clusters are both, adequate choices. N- Number

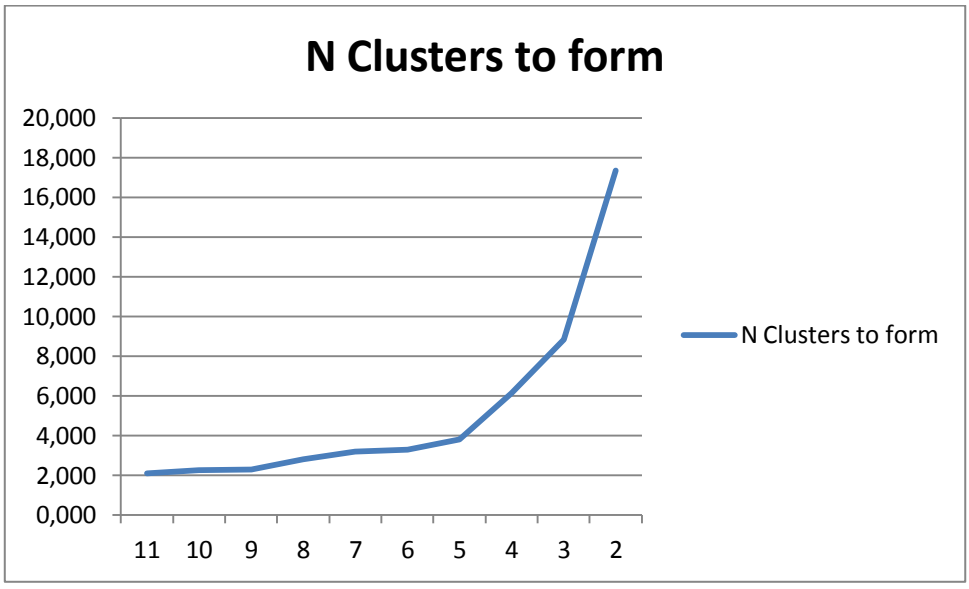


Fig. 17: Qualitative variables- the subtraction between the last 10 fusion coefficients, gaves the differences between fusion coefficients the biggest differences, indicate the more appropriate numbers of clusters to form, in this case, the biggest difference occurs in the position 2, followed by the positions 3 and 4 that are practically equivalent among themselves, therefore 2, 3 or 4 clusters are all, adequate. N- Number.

Considering the fusion coefficients distances obtained during the cluster analysis, the graphic display of those data, the information presented on the dendograms, and the nature of the sample it was decided to form 3 groups of clusters both for quantitative and qualitative variable analysis.

Therefore in order to characterize the profile of these clusters, analysis of variance (ANOVA) statistical model was performed for quantitative variables and cross-tabulation statistics was effectuated for qualitative variables.

4.1.2 Quantitative clusters analysis

Considering the objective of classifying the characteristics of the formed males' quantitative variables clusters; a one-way ANOVA statistical analysis was performed. This analysis allowed characterizing the morphological features of this male sample in relation to several descriptive measures; the table containing that information is table 3, and is it displayed as follows.

Table 3 – Males descriptive statistics of quantitative variables within the clusters formed by hierarchical cluster analysis.

Morfological feature	Cluster	Descritive measures					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Coscutum lenght/width ratio	1	85	1,481	0,131	0,014	0,985	1,903
	2	102	1,524	0,083	0,008	1,329	1,752
	3	52	1,538	0,125	0,017	1,218	1,979
	Total	239	1,512	0,114	0,007	0,985	1,979
After posteromedian groves measured width/ Coscutum width ratio	1	85	0,699	0,047	0,005	0,597	0,804
	2	102	0,760	0,050	0,005	0,625	0,865
	3	52	0,732	0,054	0,007	0,613	0,883
	Total	239	0,733	0,057	0,004	0,597	0,883
Basis capituli lenght/width ratio	1	85	0,866	0,047	0,005	0,732	0,965
	2	102	0,869	0,049	0,005	0,701	0,988
	3	52	0,911	0,048	0,007	0,802	1,100
	Total	239	0,877	0,051	0,003	0,701	1,100
Adanal plates height/width ratio	1	85	2,393	0,223	0,024	1,831	2,900
	2	102	2,333	0,320	0,031	0,749	2,987
	3	52	2,318	0,286	0,039	1,656	2,939
	Total	239	2,351	0,282	0,018	0,749	2,987
Spiracle oval area heigth/width ratio	1	85	3,319	0,611	0,066	2,075	5,041
	2	102	2,689	0,364	0,036	1,823	3,640
	3	52	2,441	0,324	0,045	1,477	3,342
	Total	239	2,859	0,580	0,038	1,477	5,041
Spiracle areas third width ratio	1	85	1,679	0,252	0,027	0,779	2,264
	2	102	2,030	0,329	0,033	1,184	3,576
	3	52	1,575	0,224	0,031	1,094	2,000
	Total	239	1,807	0,344	0,022	0,779	3,576
Spiracle ending tail width/adjacent festoon width ratio	1	85	0,418	0,083	0,009	0,217	0,652
	2	102	0,436	0,096	0,009	0,259	0,766
	3	52	0,650	0,125	0,017	0,382	0,978
	Total	239	0,476	0,135	0,910	0,217	0,978

Table 3 – (Continued)

Morfological Feature	Cluster	Descritive Feature					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Spiracle areas tail angle	1	85	128,702	11,082	1,202	100,186	149,754
	2	102	142,786	13,345	1,321	107,920	174,820
	3	52	143,219	12,262	1,700	103,662	167,394
	Total	239	137,872	14,063	0,909	100,186	174,820

Note: All measures were taken in millimeters, except the Spiracular areas tail angle taken in angle degrees. N- Number of elements within the clusters, Std. Deviation – standard deviation, Std. Error – standard error

The descriptive statistic measure used to describe these three clusters was the mean. The means of the quantitative variables within the 3 clusters formed are graphically displayed in Fig. 18 and Fig. 19. The "Spiracle areas angle" graphic is apart from the others, because this is the quantitative variable that shows the highest unit values (angle degrees), presenting the highest standard deviation between clusters. This occurence can be easily explained by the fact that it is the only variable not expressed in milimeters.

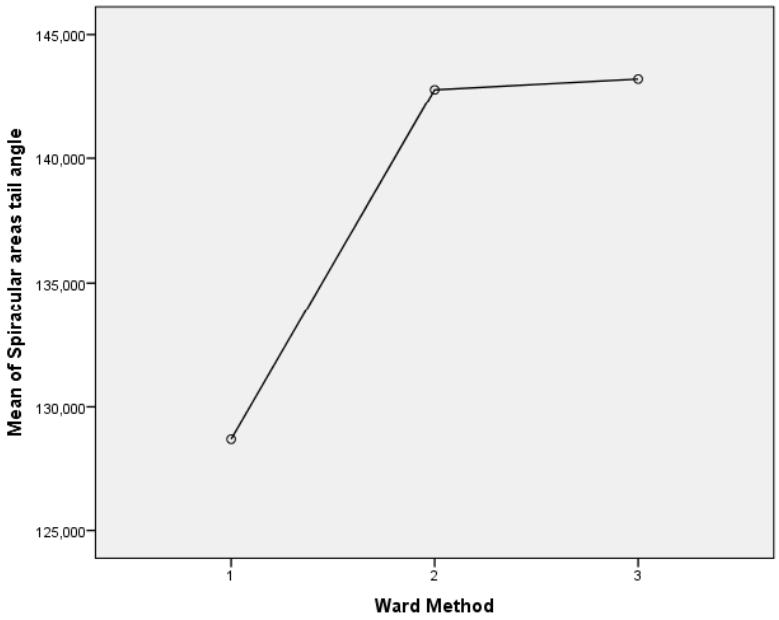


Fig. 18: "Spiracular area tail angle" quantitative variable male's clusters mean. In comparison to the others quantitative variables, this one presents the highest mean values; as a result of this variable being measured in degrees not millimeters. When compared with the others clusters, the one that is better differentiated by its mean is cluster 1, in turn the clusters 2 and 3 are poorly differentiated, since they have very similar means. The Cluster means are Cluster 1 – μ=128,703, Cluster 2 – μ=142,786 and Cluster 3 – μ=143,219.

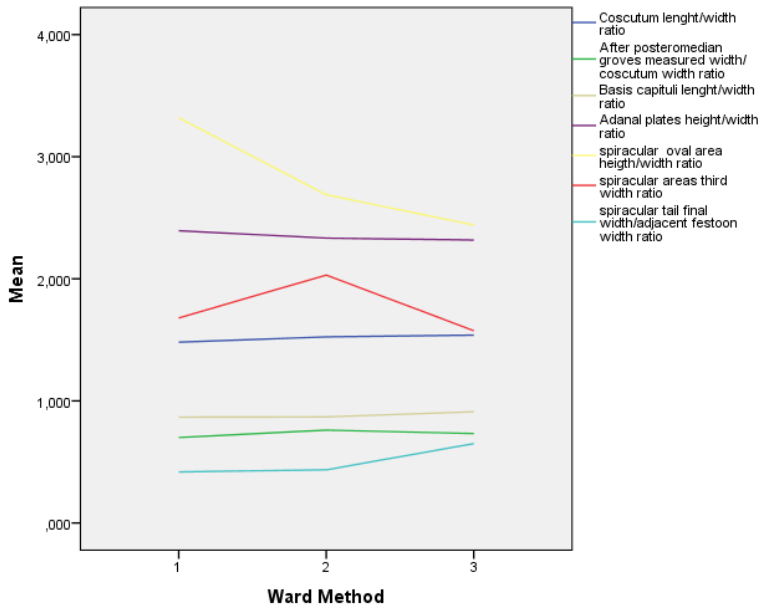


Fig. 19: Clusters averages obtained based on all males quantitative variables less the spiracular area tail angle. The variables where the means plainly defines the different clusters were "After posteromedian grooves measured width/conscutum width ratio" and the "Spiracle oval area length/Width ratio". The following variables failed to differentiate the means of two clusters "Conscutum length/width ratio" of the 2-3, "Basis capituli length/ width ratio" of the 1-2 "Spiracle tail ending width/ adjacent festoon width ratio" of the 1-2, "Spiracl areas thirds width ratio" of the 1-3 "Adanal plates height/width" of the 2-3.

Considering the data displayed on table 3 and both fig. 18 and fig. 19, the following conclusions regarding the contribution of the quantitative variables averagess to the differentiation between clusters, can be presented:

- The variables, where the means clearly defined the distinct clusters, were "After posteromedian grooves measured width/ width conscutum ratio" and "Spiracle oval area length/width ratio"

- The variables that failed to differentiate the means of two clusters were the "Conscutum length/width ratio", the "Basis capituli length/ width ratio" the "Spiracle tail ending width/ adjacent festoon width ratio", the "Spiracle areas thirds width ratio" the "Adanal plates height/width and "Spiracle area tail angle". These variables did not differentiated the means of 2-3, 1-2, 1-2, 2-3, 2-3 and 2-3 clusters, respectively.

Considering the results above the following characterization for the 3 distinct clusters can be presented:

- The cluster 1 presents the elements with the largest conscutums and also the largest capitulli of this sample, this cluster also presents big adanal plates, the longest and narrower spiracles, with the smallest tail angle of the 3 clusters formed, and the ending width of the spiracle tail, is less than half than the width of the adjacent festoon.

- The cluster 2 contains mostly specimens with average size conscutums, being particularly wide in the region after the posteromedian grooves. This cluster also displays medium sized capitulli, big adanal plates, spiracles presenting big tail angles, average length, large widths, and the ending width of the spiracle tail is less than half than the width of the adjacent festoon.

- The cluster 3 presents individuals with the smallest conscutums, the smallest capitulli and the smallest adanal plates among the population considered for this study. This cluster also displays spiracles with big tail angles, short lengths, large widths, and the ending width of the spiracle tail is more than half than the width of the adjacent festoon.

With the purpose of classifying the statistical significance of the obtained results, an ANOVA was effectuated. The following values were acquired:

- The "Adanal plates height/width ratio" variable results were p=0,219 and F=1,528, this indicates that the variable did not significantly differentiated the means between clusters.

- The "Spiracle tail ending width/adjacent festoon width ratio" variable results were p=0,000 and F=103,254, the variable "Spiracle oval area height/width ratio" presented the results; p=0,000 and F= 70,502, the "Spiracle area thirds-widths ratio" variable results were p=0,000 and F= 58,140, the results of the variable "Spiracle tail angle" were p=0,000 and F= 36,400, the variable " After posteromedian grooves measured width/conscutum width ratio" presented the results p=0,000 and F= 33,970, the results of the variable "Basis capituli length/width ratio" were p=0,000 and F=16,457, finally the "Conscutum lenght/width ratio" variable results were p= 0,006 e F= 5,316.

- As consequence, all this variables statistical significantly differentiated the clusters means, this is, all of them gave a significant contribute for the clusters formation. Considering the F-value, the variables are in descending order of significance for the cluster formation,

meaning that the variable that more significantly contributed for the clusters formation was the "Spiracle ending tail width/adjacent festoon width ratio" and in turn the variable that less significantly contributed for the cluster formation was the "Conscutum length/width ratio".

Usually used in conjunction with ANOVA, a multiple comparison Tukey HSD test (post hoc test) was performed. It is a single-step multiple comparison procedure, that works as a statistical tool, permiting to find the averages that are significantly different from each other. This test allowed obtaining of the following results:

- The "Adanal plates height/width ratio" variable presented results that did not fulfilled the condition of the test: H0 hypothesis ($p \leq 0{,}050$), as consequence, it cannot be evaluated by this test, and it is not statistically significant for the differentiation of the clusters averages.

- Despite some statistically significant p-values were observed; the "Conscutum length/ width ratio" variable did not presented a statically significant difference between the 2-3 cluster mean ($p=0{,}741$); the variable "Basis Capituli length/width ratio" did not presented a statistically significant difference between the 1-2 clusters mean ($p=0{,}954$); "Spiracle area thirds-widths ratio" variable did not presented a statistically significant difference between 1-3 clusters mean ($p=0{,}093$); the "Spiracle tail ending width/adjacent festoon width ratio" variable did not presented a statistically difference between the 1-2 clusters mean ($p=0{,}460$); the "Spiracle area tail angle" variable did not presented a statistically significant difference between 2-3 clusters mean ($p=0{,}977$).

- The variables, "Spiracle area height/width ratio" and "After posteromedian grooves measured width/conscutum width ratio" are the ones that gave the highest contribute to the clusters formation. These variables are both able to statistically differentiate with significance all the clusters averages, once they both present exclusively $p<0{,}050$ between the 3 clusters.

4.1.3 Qualitative Variable clusters analysis

A cross-tabulation statistics was performed in order to classify the formed males' qualitative variables clusters, from the 239 males present on this study. The males' qualitative variables clusters characterization, by percentage, is described for each of the 3 clusters that information is presented in appendices in pages 143 to 145.

The results on the association measure Cramer's V and Chi-square test were acquired for all variables relatively to the qualitative variables groups, and are presented as follows:

- The variables "Cervical fields depression", "Cervical fields shape", "Cervical setiferous punctuations", "Capituli ventral palps height", "Palp shape (2^{nd}A)", "Lateral grooves festoon ending", "Lateral grooves texture", "Paramedian grooves shape" and "Adanal plates total shape", failed to meet the test condition (<20% of cells with expected count less than 5 and minimum expected counts higher that 1) therefore they will not be interpreted.

- The "Parma presence" variable result was p=0,000 ($\chi2(1)$=204,090) and V=0,924, what indicates that this variable has a statistically significant relation and once that this variable presents the highest V-value, it is the main variable for the qualitative variables groups formation.

- The "Adanal plates ending" variable results were p=0,000 ($\chi2(1)$=44,779 and V=0,433 and the results of the variable "Posteromedian grooves short or long" were p=0,000 ($\chi2(1)$=35,929 and V= 0,384, therefore both variables have moderate statistical significant effect on the qualitative clusters formation.

- The "Conscutum punctuation size" variable results were p=0,000 ($\chi2(1)$=20,961 and V=0,209, the "Cervical groove definition" variable results were p= 0,006 ($\chi2(1)$=10,161 and V=0,206, the results of the variable "Spiracle area type" were p=0,010 ($\chi2(1)$=19,422 and V=0,202, the variable "Paramedian grooves deepness" results were p=0,010 ($\chi2(1)$=9,210 and V=0,196, and the "Conscutum punctuation distribution" results were p=0,045 ($\chi2(1)$=6,217 and V=0,160, which tell us that these variables have a low statistical significant effect on the qualitative clusters formation. Considering the V-value

these variables are in descending order of contribution for the qualitative clusters formation.

- The "Posteromedian grooves deepness" variables results were p=0,538 $\chi 2(1)=1,124$ and V=0,072, the "Adanal plates posterior margin" results were p=0,597 $\chi 2(1)=1,031$ and V=0,066, and the results of the variable "Lateral grooves beginning" were p=0,806 $\chi 2(1)=0,933$ and V=0,043. These results indicate that these variables do not have a statistical significant effect on the qualitative clusters formation.

If we look at the results obtained so far, it is possible to infer that the quantitative variables that gave the most contribute to the quantitative cluster formation were "Spiracle ending tail width/adjacent festoon final width ratio" and "Spiracle oval area height/width ratio" followed by the variables "Spiracle third-widths ratio", "Spiracle area tail angle", "After posteromedian grooves measured width/conscutum width ratio" and "Basis capituli length/width ratio". Among the variables that statistically contributed for the quantitative clusters formation the one that less contributed was "Conscutum length/width ratio". The results also indicate that the variable "Adanal plates height/width ratio" did not contribute for the quantitative clusters formations.

As to the qualitative clusters formation, it is possible to say that the variable that had the most statistical significant effect on the qualitative clusters formation was "Parma presence", the variables "Posteromedian grooves short or long" and "Conscutum punctuation size" both have a moderate statistical significant effect on the quantitative clusters formation. The results also indicate that the variables "Conscutum punctuation size", "Spiracle area type", "Paramedian grooves deepness" and "Conscutum punctuation distribution", also contribute for the qualitative clusters formation although, they all have a weak statistical significant effect on cluster formation, the results of the analysis performed so far also indicates that, all the other qualitative variables do not have a statistical significant effect on the quantitative clusters formation.

4.1.4 Correspondence analysis

Taking into account, that the data presented on this study contain both quantitative and qualitative variables that lead to the formation of two types of clusters, in order to achieve

conclusions, concerning the associations between the quantitative and qualitative clusters, it was decided to resort to correspondence analysis. That is an exploratory data technique, used to analyze the association of two or more categorical variables, therefore allowing data reduction and graphical representation (bivariate graph) of dissimilarities on categorical variables.

So, as mean to achieve this purpose, the correspondence analysis was performed on the quantitative variables and qualitative variables previously formed clusters, that lead to obtaining the results, inertia value=0,070, as it is one adequate result, if we consider that a total above 0,20 must be obtained in other to acquire proper representations, and chi-square test (p=0,002 and $\chi 2(1)=16,646$). These results indicate the presence of a significant statistical correlation between both variables, as result the output presented in fig. 20 reveals the presence of associations among the clusters 2 of both typologies. The same situation occurs between the clusters 3 of both typologies, there is also association among the cluster 1 of the qualitative typology and clusters 2 of quantitative typology, however the strongest association occurs between the cluster 1 of the quantitative typology and the cluster 2 of the qualitative typology.

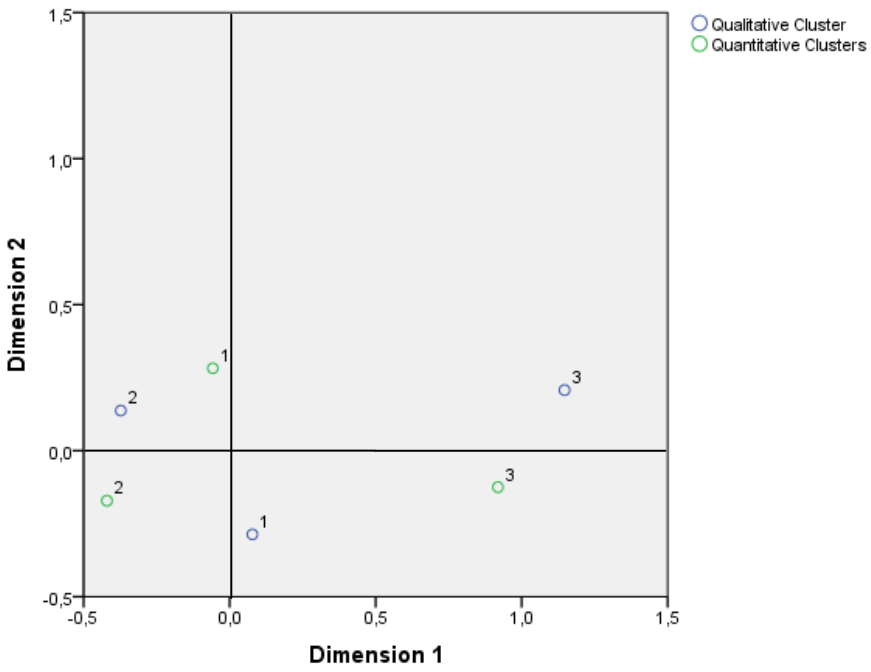


Fig. 20: Bivariate graph acquired from correspondence analysis of the quantitative variables with the qualitative variables of males formed clusters. The results I=0,070 and p=0,002 suggest the presence of a statistical significant correlation between the both types of variables considered. Data presented on this bivariate graph evidences the associations between the clusters 2 of both typologies, the same situation occur between both clusters 3. There are all also associations between clusters 1 of the qualitative typology and the cluster 2 of the quantitative typology; and cluster 2 of the qualitative typology and cluster 3 of the quantitative typology.

4.1.5 Morphologic Classification

Alongside with the hierarchical cluster analysis effectuated for quantitative and qualitative variables, conducted in SSPS software [96], a morphologic analysis, was performed in all 239 males contained in our sample.

So using these criteria, it was observed that our sample contained specimens that belong to the following morphologic groups: *R. sanguineus sensu stricto* (morphology similar to the african specimens, which will be designated as africanus or Af), *R. sanguineus* T1, *R. sanguineus* T2, *R. turanicus*, *R. pusillus*, and also 3 other morphological groups that have not been described before it was decided to name then: *R. sanguineus* D, *R. sanguineus* R, and *R. turanicus D*.

Among this sample, only the males exhibiting typical characteristics of the *R. sanguineus* s. s. species such as spiracles with long and narrow tails, were classified as such and the same criteria was applied to *R. turanicus*, once only the specimens presenting spiracles with short and wide tails, were distinguished as such. These rigid criteria has led to great part of the sample to be classified as intermediate forms, mostly *R. sanguineus* T1 and *R. sanguineus* T2.

As so *R. sanguineus* Type 1 refers to intermediate forms which have in common more characteristics with *R. sanguineus*, than with *R. turanicus*, for example despite their spiracular region, not having such a high and narrow termination as the one seen in *R. sanguineus*, it still has plenty of features similar to that species; the difference between, this specimens and the ones classified as *R. sanguineus* Type 2 is that, the type 2 typology shares less characteristics with *R. sanguineus*, namely the body of their spiracle is not so narrow and is shaped like a globe. Also the termination of the tail significantly shorter and wider than the one presented by *R. sanguineus* s. l. as consequence *R. sanguineus* Type 2 is more morphological distant to *R. sanguineus s. s.* than the type 1 typology.

R. sanguineus R, typology refers to ticks that display spiracles with very narrow tails and very large bodies, *R. sanguineus* D, typology refers to elements, which have spiracles were a very gradual transition between the dimensions of the tail and body of the spiracle occurs. The same situation was observed in ticks that clearly, presented features, of the *R. turanicus* species, these elements compose the *R. turanicus* D, morphologic group. Our sample also

displays some specimens that belong to the species *R. pusillus*, which were included in this study as an outlier in order to function as a control.
Note: All the 8 morphologic groups mentioned in this paragraph are, detailed described, with text and images in pages 57 to 59.

The 8 morphologic groups observed in this sample can be converted in morphologic clusters, in order, to be analyzed alongside with the qualitative and quantitative cluster previously formed.

The information of each male element regarding the taxonomic group to which it belongs as well as the quanlitative and quantitative clusters, where it was previously inserted alongside with the region where it was collected and the identifying number of eachs specimen is displayed in table 4 presented in appendices page 148.

By taking a closer look at the information displayed at table 3, it is easily remarkable that there are several points of possible analysis, namely by studying the region were the specimens were collected. So, in the 239 males considered for this study, 70 (29,3%) specimens were collected in Setúbal, eight (3,4%) in Alcochete, six (2,5%) in Peniche, 33 (13,4%) in Alcobaça and 122 (51,0%) in Vila Franca de Xira. The geographic distribution of the specimens will not be considered in this study, due to the low number of specimens collected specially in Peniche and Alcochete (Fig 21).

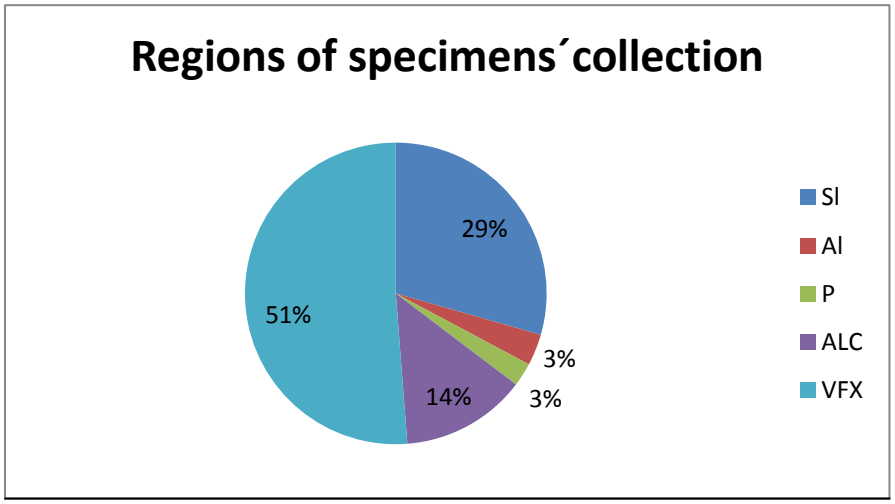


Fig. 21: Regions of specimens' collection- Graphic summarizing the information relative to the amount of male specimens collected in each region. **Sl:** Setúbal – 70 males (29,3%); **Al:** Alcochete – 8 males (3,4%); **P:** Peniche – 6 males (2,5%); **ALC:** Alcobaça – 33 males (13,4%); **VFX:** Vila Franca de Xira – 122 males (51,0%)

Table 4 also provides information about the morphological cluster that each specimen belongs. By analyzing the sample based on this parameter it is possible to state that: in the 239 males, 28 (11,7%) were classified as *R. sanguineus sensu stricto*, 81 (33,9%) as *R. sanguineus* type 1, 74 (30,9%) as *R. sanguineus* type 2, 11 (4,6%) as *R. sanguineus* D, five (2,1%) as *R. sanguineus* R, 27 (11,3 %) as *R. turanicus*, three (1,3%) as *R. turanicus* D and 10 (4,2) as *R. pusillus* (Fig.22).

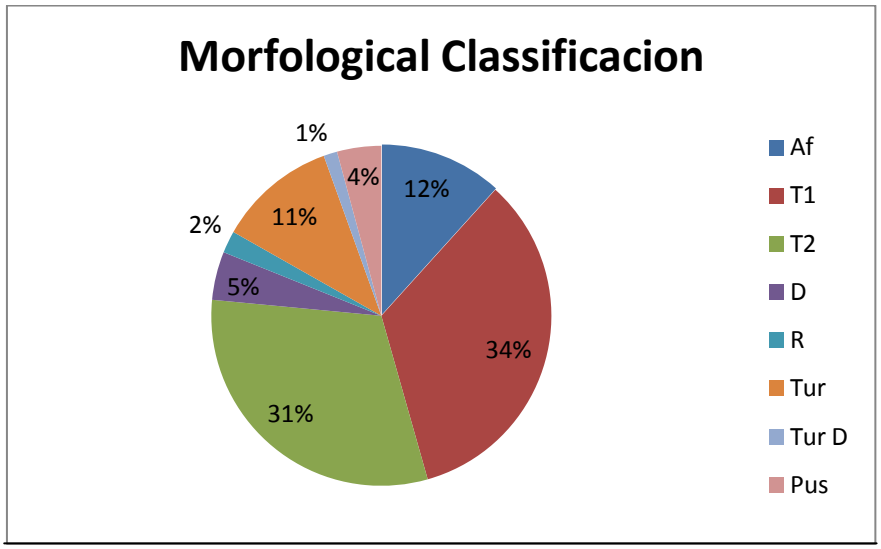


Fig. 22: Morphologic Classification- Graphic that synthesizes the information, relative to the morphological classification within the sample considered in this study**: Af:** *R. sanguineus sensu stricto* – 28 males (11,7%); **T1:** *R. sanguineus* Type 1 – 81 males (33,9%); **T2:** *R. sanguineus* type 2 – 74 males (30,9%); **D:** *R. sanguineus* D – 11 males (4,6%); **R:** *R. sanguineus* R – 5 males (2,1%); **Tur:** *R. turanicus* – 27 males (11,3%); **Tur D:** *R. turanicus D* – 3 males (1,3%); **Pus:** *R. pusillus* – 10 males (4,2%).

Alongside with the information about the morphological cluster that each specimen belongs, table 4, also displays the same information, concerning to the qualitative and qualitative clusters that each male tick belongs. Therefore it is possible to relate morphological clusters with quantitative and qualitative clusters respectively.

In order to understand what are the morphological groups that appear most frequently associated with each of the quantitative clusters, and also how this relationship occurs in the qualitative clusters, it is necessary, to know what morphological characteristics are presented by each morphological clusters. This information is exposed in Table 5.

Table 5 – Males descriptive statistics of quantitative variables within the morphologic clusters.

Morphological Feature	Morphologic Cluster	Descritive measures					
		N	Average	Std. Deviation	Std. Error	Minimum	Maximum
Coscutum lenght/width ratio	T1	80	1,514	0,106	0,012	1,316	1,903
	T2	74	1,514	0,115	0,013	0,985	1,716
	Af	28	1,513	0,137	0,026	1,270	1,854
	Tur	27	1,503	0,136	0,026	1,218	1,979
	Pus	10	1,512	0,075	0,024	1,334	1,624
	D	12	1,481	0,117	0,034	1,227	1,624
	R	5	1,554	0,027	0,012	1,512	1,579
	Tur D	3	1,536	0,008	0,004	1,529	1,544
	Total	239	1,512	0,114	0,007	0,985	1,979
After posteromedian groves measured width/ Coscutum width ratio	T1	80	0,734	0,059	0,007	0,597	0,858
	T2	74	0,738	0,057	0,007	0,605	0,865
	Af	28	0,708	0,039	0,007	0,644	0,796
	Tur	27	0,731	0,061	0,012	0,613	0,883
	Pus	10	0,753	0,035	0,011	0,696	0,789
	D	12	0,722	0,067	0,019	0,616	0,843
	R	5	0,783	0,040	0,018	0,731	0,824
	Tur D	3	0,741	0,033	0,019	0,708	0,774
	Total	239	0,733	0,057	0,004	0,597	0,883
Basis capituli lenght/width ratio	T1	80	0,876	0,049	0,005	0,701	0,988
	T2	74	0,867	0,058	0,007	0,742	1,100
	Af	28	0,871	0,042	0,008	0,759	0,964
	Tur	27	0,903	0,042	0,008	0,826	0,977
	Pus	10	0,930	0,035	0,011	0,889	0,977
	D	12	0,858	0,038	0,011	0,820	0,946
	R	5	0,859	0,047	0,021	0,789	0,918
	Tur D	3	0,911	0,026	0,015	0,882	0,931
	Total	239	0,877	0,051	0,003	0,701	1,100
Adanal plates height/width ratio	T1	80	2,358	0,261	0,029	1,095	2,863
	T2	74	2,341	0,319	0,037	0,749	2,987
	Af	28	2,412	0,180	0,034	2,090	2,853
	Tur	27	2,359	0,230	0,044	1,947	2,901
	Pus	10	2,038	0,337	0,107	1,656	2,644
	D	12	2,399	0,285	0,082	1,974	2,900
	R	5	2,291	0,294	0,131	1,985	2,743
	Tur D	3	2,725	0,215	0,124	2,509	2,939
	Total	239	2,351	0,282	0,018	0,749	2,987
Spiracle oval area heigth/width ratio	T1	80	2,910	0,491	0,055	1,823	4,140
	T2	74	2,657	0,331	0,039	1,931	3,885
	Af	28	3,810	0,608	0,115	2,313	5,041

Table 5 – (Continued)

Morphological Feature	Morphologic Cluster	Descriptive Measure					
		N	Average	Std. Desviation	Std. Error	Minimum	Maximum
Spiracle oval area heigth/width ratio	Tur	27	2,446	0,337	0,065	1,904	3,075
	Pus	10	2,409	0,100	0,032	2,250	2,568
	D	12	3,055	0,312	0,090	2,433	3,613
	R	5	2,687	0,586	0,262	2,258	3,589
	Tur D	3	2,320	0,737	0,425	1,477	2,840
	Total	239	2,859	0,581	0,038	1,477	5,041
Spiracle areas third width ratio	T1	80	1,912	0,371	0,042	0,779	3,576
	T2	74	1,903	0,248	0,029	1,342	2,437
	Af	28	1,677	0,250	0,047	1,297	2,264
	Tur	27	1,551	0,184	0,035	1,184	1,896
	Pus	10	1,408	0,147	0,046	1,094	1,581
	D	12	1,572	0,208	0,060	1,304	1,976
	R	5	2,352	0,546	0,244	1,859	3,213
	Tur D	3	1,475	0,446	0,257	1,110	1,972
	Total	239	1,807	0,344	0,022	0,779	3,576
Spiracle ending tail width/adjacent festoon width ratio	T1	80	0,415	0,080	0,009	0,259	0,711
	T2	74	0,474	0,096	0,011	0,283	0,766
	Af	28	0,367	0,075	0,014	0,217	0,520
	Tur	27	0,677	0,113	0,022	0,516	0,978
	Pus	10	0,736	0,077	0,024	0,676	0,943
	D	12	0,470	0,072	0,021	0,331	0,600
	R	5	0,411	0,116	0,052	0,275	0,537
	Tur D	3	0,636	0,083	0,048	0,578	0,730
	Total	239	0,476	0,135	0,009	0,217	0,978
Spiracle areas tail angle	T1	80	136,511	13,425	1,501	107,635	172,890
	T2	74	138,040	14,903	1,732	100,186	164,915
	Af	28	131,213	10,959	2,071	114,376	164,475
	Tur	27	143,057	13,046	2,511	121,642	167,394
	Pus	10	140,257	10,594	3,350	126,062	156,650
	D	12	135,280	10,381	2,997	116,889	150,709
	R	5	166,570	8,794	3,933	153,499	174,820
	Tur D	3,000	140,056	5,230	3,020	135,102	145,524
	Total	239	137,872	14,063	0,910	100,186	174,820

Note: All measures were taken in millimeters, except the Spiracular areas tail angle taken in angle degrees. **N-** Number of elements within the clusters, **Std. Deviation** – standard deviation, **Std. Error –** standard error.

Considering the results above, the following characterization for the 8 distinct morphologic clusters can be presented:

- *R. sanguineus* T1 morphologic cluster presents elements with large conscutums and large capitulli. This cluster also presents big adanal plates, long and narrow spiracles, with average tail angle, and the final width of the tail is less than half than the width of the adjacent festoon.

- *R. sanguineus* T2 morphologic cluster contains mostly specimens with average size conscutum. This cluster also displays medium sized capitulli, average sized adanal plates, spiracles presenting average tail angles, average length, large widths, and the final width of the tail is less than half than the width of the adjacent festoon, but wider than what is observed in *R. sanguineus* T1.

- *R. sanguineus* af morphologic cluster presents the elements with the largest conscutums and also the largest capitulli of this sample. This cluster also evidences big adanal plates, the longest and narrower spiracles, with the smallest tail angle of the 8 clusters formed, and the final width of the tail is less than half of the adjacent festoon width, presenting in fact the thinner ending tail of the studied sample.

- *R. turanicus* morphologic cluster contains mostly specimens with average size conscutums. This cluster also displays medium sized capitulli, slightly under average adanal plates, spiracles with big tail angles, short length, large widths, and the final width of the tail superior to half of the of the adjacent festoon width.

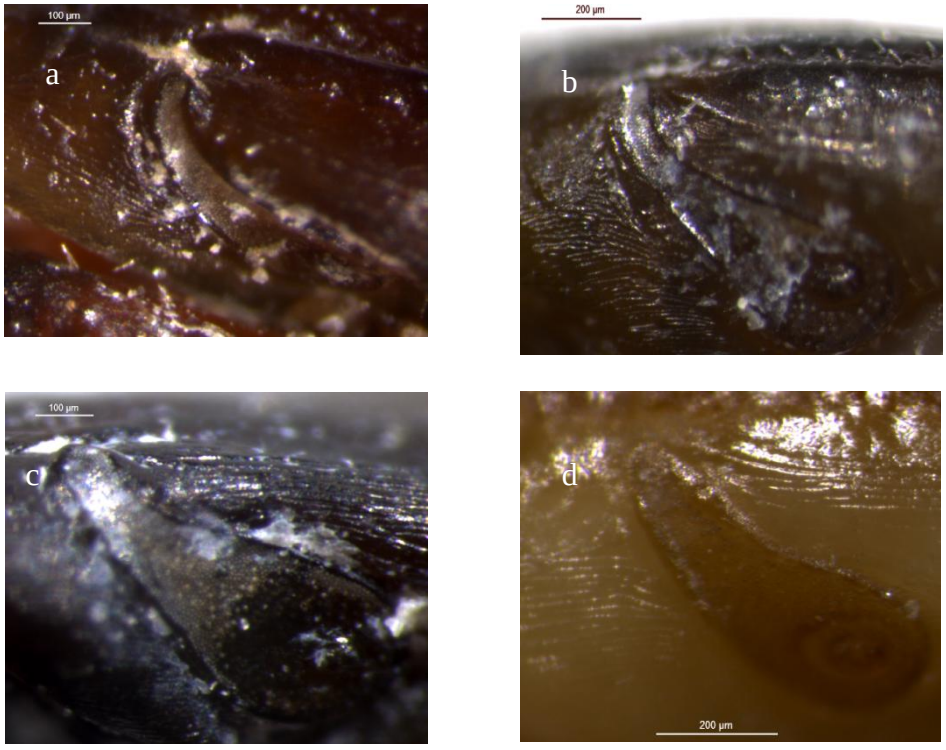
- *R. pusillus* morphologic cluster shows individuals with the smallest conscutums, the smallest capitulli and the smallest adanal plates among the population considered for this study. This cluster also displays the shortest spiracles, with high tail angles, short lengths large widths, and the final width of the tail is larger than half of the adjacent festoon width, being the morphologic cluster with the wider ending tail.

- *R. sanguineus* D morphologic cluster presents elements with large conscutums and large capitulli. This cluster also presents big adanal plates, and spiracles with a gradual dimension transition between the body and tail, which results in long and narrow spiracles, with small tail angle, and the final width of the tail is inferior to half of the adjacent festoon width, but wider than what is observed in *R. Sanguineus* T1.

- *R. sanguineus* R morphologic cluster contains mostly specimens with average sized conscutum. This cluster also displays medium sized capitulli, average sized adanal plates, spiracles presenting the higher tail angles of the 8 clusters formed, short length, large widths, therefore the spiracle has a globular body and a very thin tail that results in the final width of the tail being less than half of the adjacent festoon width, being alongside with *R. sanguineus* af the cluster that presents the thinner ending tail of the sample.

- *R. turanicus* D morphologic cluster mostly contains specimens with small sized conscutums, medium sized capitulli, slightly under average adanal plates, spiracles with big tail angles, short length, thin widths, and the final width of the tail is superior to half of the adjacent festoon width, but thinner than what is observed in *R. turanicus*, assuming a type of spiracles characterized by a regular narrowing towards the dorsal end of the tail.

It is possible to conclude then when it cames to males the main morphologic diference between the 8 morphologic clusters formed is the structure of the spiracle, those differences are detailed evidenced in fig. 23.



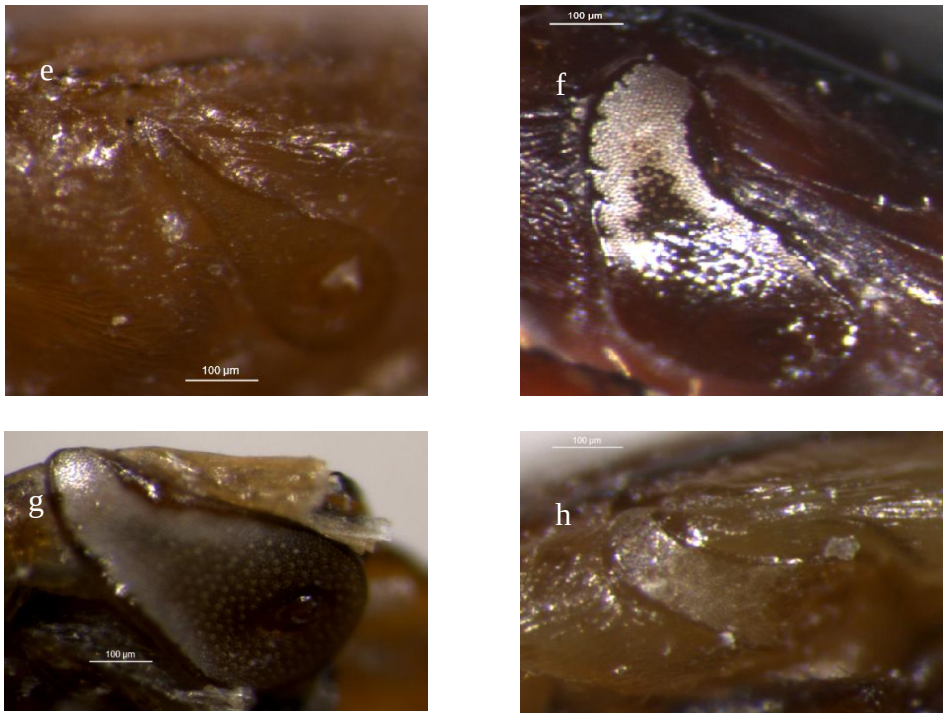


Fig. 23: Differences of morphological types of male spiracular plates identified: a. *Rhipicephalus sanguineus* s.s. (africanus); b. *R. sanguineus* type I; c- *R. sanguineus* type II; d - *R. sanguineus* D; e - *R. sanguineus* R; f - *R.* turanicus; g - R. turanicus D; h – *R. pusillus.*

By analyzing individually each quantitative cluster, it is possible to observe that, within the 85 specimens belonging to the quantitative cluster 1, 26 (30,6%) were classified as *R. sanguineus* af, 34 (40,0%) as *R. sanguineus* Type 1, 17 (20,0%) as *R. sanguineus* Type 2, and eight (9,4%) as *R. sanguineus* D (Fig. 24).

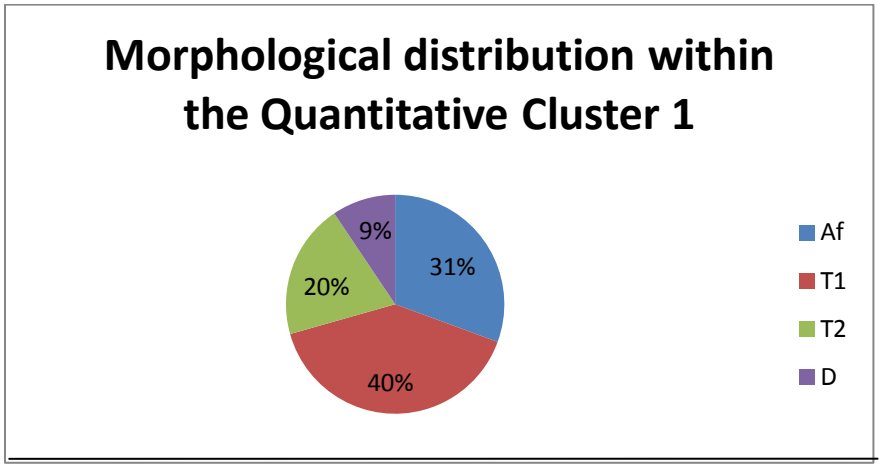


Fig. 24: Morphologic distribution within the Quantitative Cluster 1- Graphic that relates the taxonomic clusters obtained by morphologic analysis with the quantitative cluster 1 obtained by statistical analysis**: Af:** *R. sanguineus s. s.* – 26 males (30,6%); **T1:** *R. sanguineus* Type 1 – 34 males (40,0%); **T2:** *R sanguineus* type 2 – 17 males (20,6%); **D:** *R. sanguineus* D – 8 males (9,4%).

Data presented on Fig. 24, suggest that there are only four morphological groups in this cluster and within those four, where *R. sanguineus sensu stricto* (Africanus*)* and *R. sanguineus* T1 dominate over the others, it is possible to conclude that there is an association among both these morphologic clusters and quantitative cluster 1.

This association comes out as a natural result. By looking at the data presented on table 3 and at the morphological characteristics it is possible to note that quantitative cluster 1 is associated with individuals that present large conscutums, large capitulli, big adanal plates, long and narrow spiracles, with small tail angle, and the final width of the tail is inferior to half of the adjacent festoon width. All these features can be found in the morphologic clusters *R. sanguineus sensu stricto* and *R. sanguineus* T1, as evidenced in the data presented by Table 5 and by its clusters morphological descriptions.

Some of the morphologic features, presented by the morphologic clusters *R. sanguineus s. s.* and *R. sanguineus* T1, are also displayed by *R. sanguineus* D and *R. sanguineus* II. This fact explains why elements of these morphologic groups are presented in the quantitative cluster 1, although with few specimens, which shows a weaker association between these morphologic clusters and qualitative cluster 1. As both these morphologic clusters share less morphologic features with this quantitative cluster than for example *R. sanguineus s. s.* and *R. sanguineus* T1.

The information provided in table 4 shows that within the 102 specimens belonging to the quantitative cluster 2, two (2,0%) were classified as *R. sanguineus sensu stricto*, 43 (42,1%) as *R. sanguineus* Type 1, 47 (46,0%) as *R. sanguineus* Type 2, three (2,9%) as *R. sanguineus* D, five (4,9%) as *R. sanguineus* R and two (2,0%) as *R. turanicus* (Fig. 25).

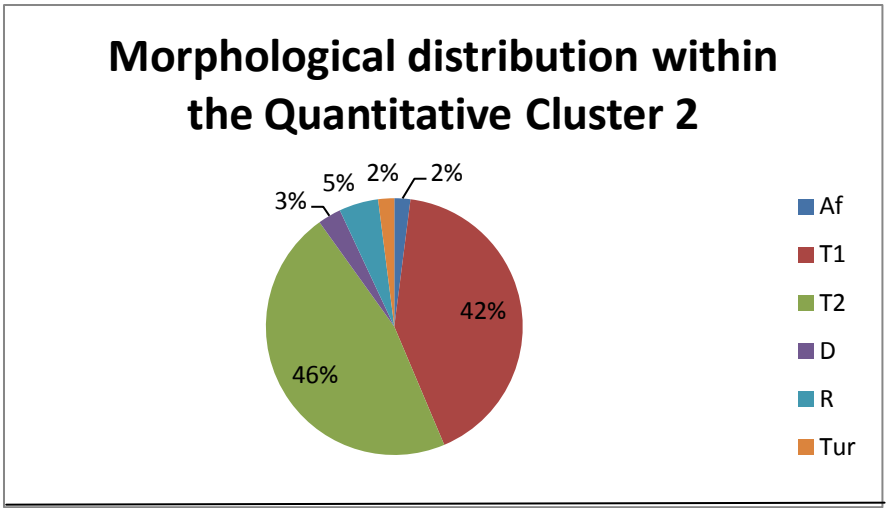


Fig. 25: Morphologic distribution within the Quantitative Cluster 2- Graphic that relates the taxonomic clusters obtained by morphologic analysis, with the qualitative cluster 2 by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 2 males (2,0%); **T1:** *R. sanguineus* Type 1 – 43 males (42,1%); **T2:** *R sanguineus* type 2 – 47 males (46,0%); **D:** *R. sanguineus* D – 3 males (2,9%); **R:** *R. sanguineus* R – 5 males (4,9,%); **Tur:** *R. turanicus* – 2 males (2,0%).

Data presented on Fig. 25, shows the presence of six different morphological groups within the quantitative cluster 2. The presence of so many morphological groups, as distinct from each other such as *R. sanguineus* and *R. turanicus* suggests this is a quantitative cluster, with large intra-specific variation, and therefore it is difficult to define the morphological characteristics that prevail.

Nevertheless there are two groups, *R. sanguineus* T1 and *R. sanguineus* T2 that clearly dominate over the others. This fact indicates an association between both these morphological clusters and this quantitative cluster, which may explain the presence of many morphologic groups within the quantitative Cluster 2, since it is associated, with *R. sanguineus* T1 and *R. sanguineus* T2 which are two intermediate forms that share some morphological characteristics with *R. sanguineus* and *R. turanicus.* This allows the formation of a cluster characterized by a wide range of features, which permits the inclusion of such distinctive morphological groups.

The association between the quantitative Cluster 2 and the morphologic clusters, *R. sanguineus* T1 and *R. sanguineus* T2, comes out as a logical result, because quantitative cluster 2 mostly presents, specimens with average, medium sized capitulli, big adanal plates, spiracles presenting big tail angles, average length and large widths, and the final width of the tail is inferior to half of the adjacent festoon width. These morphological characteristics are

consistent with the data relative to *R. sanguineus* T1 and *R. sanguineus* T2 presented in table 5 and with its descriptions.

When looking at Table 3, namely the variable, "Spiracular ending tail width/adjacent festoon width ratio" is possible to notice that quantitative cluster 1 is associated with the value 0,418 and quantitative cluster 2 in turn is associated to the value of 0,436, this difference though subtle, is sufficient to suggest that quantitative cluster 2 is associated with specimens that present spiracles with wider terminations of the tail. This may explain why this cluster has a decreased number of *R. sanguineus sensu stricto* and increased number of *R. sanguineus* T2, when compared with the quantitative cluster 1 which is associated with narrower spiracles and with thinner terminations of the tail.

In turn, data showwithin the 52 specimens belonging to the quantitative cluster 3, four (7,7%) were classified as *R. sanguineus* type 1, 10 (19,2%) as *R. sanguineus* Type 2, 25 (48,1%) as *R. turanicus*, three (5,8%) as *R. turanicus* D, and 10 (19,2) as *R. pusillus* (Fig 26).

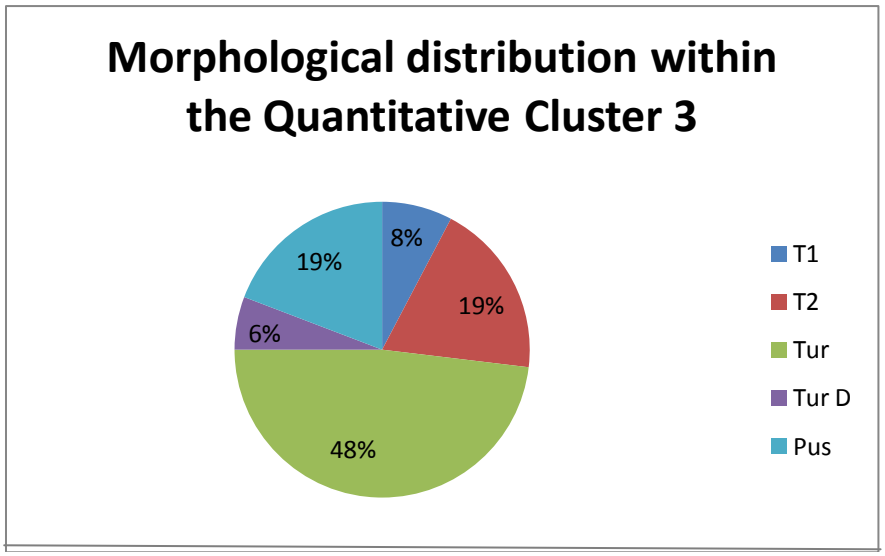


Fig. 26: Morphologic distribution within the Quantitative Cluster 3- Graphic that relates the taxonomic clusters obtained by morphologic analysis, with the quantitative cluster 3 obtained by statistical analysis**: T1:** *R. sanguineus* Type 1 – 4 males (7,7%); **T2:** *R sanguineus* type 2 – 10 males (19,9%); **Tur:** *R. turanicus* – 25 males (48,1%); **Tur D:** *R. turanicus D* – 3 males (5,8%); **Pus:** *R. pusillus* – 10 males (14,2%).

Data presented on Fig. 26, suggest that there are five morphological groups in this cluster and *R. turanicus* prevails over all the others. So it is possible to conclude that there is an association between these morphologic cluster and quantitative cluster 3. In fact, if we compare the dominant morphological groups in each quantitative cluster, the dominance of

the *R. turanicus* morphologic cluster within the quantitative cluster 3 is more intense, that as had previously been seen in quantitative cluster 1 and quantitative cluster 2 respectively. Thus, this suggests that the association between the *R. turanicus* morphologic cluster and quantitative cluster 3 is the strongest association between a quantitative cluster and a morphologic cluster observed in this study.

This association is supported by the fact that this quantitative cluster is associated with elements that present small conscutums, small capitulli and small adanal plates. This cluster also displays spiracles with big tail angles, short lengths large widths, and the final width of the tail is superior to half of the adjacent festoon width. Some of these morphologic features are consistent with the data related to *R. turanicus* presented in table 4 and with the description of this morphological cluster.

Data displayed in fig 26 also enlightens the reason why quantitative cluster 3 is the one with the smallest conscutums, the smallest capitulli, and the smallest adanal plates of the sample considered in this study. All the elements that belong to the *R. pusillus* species were included in this cluster, as this species is characterized by having smaller dimensions of the various structures that composes its bodies. The inclusion of these elements in the cluster led to a significant lowering of the dimensions mean of certain morphological characteristics.

Though, the specimens belonging to the *R. pusillus* species were included in this cluster, mostly due to the morphology of their spiracles, showing big tail angles, short lengths, large widths, and the final width of the tail is superior to half of the adjacent festoon width. These features were similar to those that can be find in *R. turanicus*, which explains why these two morphologic groups are associated with quantitative cluster 3. In fact, if we look at the morphologic clusters presented in this quantitative cluster, apart from some outliers belonging to *R. sanguineus* type 1, all the others, *R. turanicus, R. pusillus, R. turanicus* D and sometimes even *R. sanguineus* type 2, evidence spiracular areas with the characteristics described above.

By performing a similar analysis, but this time considering qualitative and morphological clusters is also possible to reach some conclusions regarding to the morphological groups that appears to be most frequently associated with each of the qualitative clusters, and also, how this relationship occurs in the qualitative clusters.

Data show that within the 83 specimens belonging to the qualitative cluster 1, nine (10,8%) were classified as *R. sanguineus sensu stricto*, 28 (33,7%) as *R. sanguineus* Type 1, 27 (32,5%) as *R. sanguineus* Type 2, two (2,4%) as *R. sanguineus* D, two (2,4%) as *R. sanguineus* R, 12 (14,6%) as *R. turanicus*, one(1,2%) as *R. turanicus* D and two (2,4%) as *R. pusillus* (Fig 27).

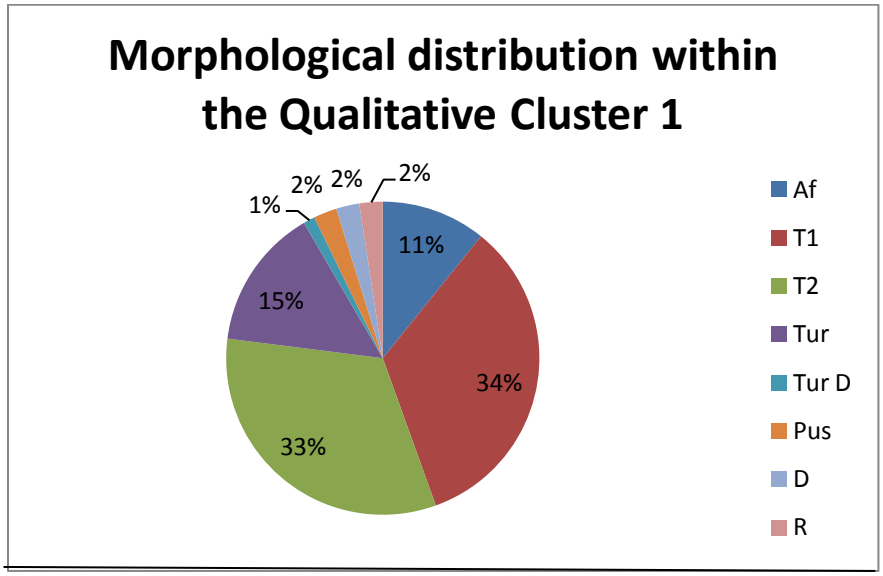


Fig. 27: Morphologic distribution within the Qualitative Cluster 1- Graphic that relates the morphologic clusters obtained by morphologic analysis, with the qualitative cluster 1 by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 9 males (10,8%); **T1:** *R. sanguineus* Type 1 – 28 males (33,7%); **T2:** *R sanguineus* type 2 – 27 males (32,5%); **D:** *R. sanguineus* D – 2 males (2,4%); **R:** *R. sanguineus* R – 2 males (2,4%); **Tur:** *R. turanicus* – 12 males (14,6%); **Tur D:** *R. turanicus D* – 1 males (1,2%); **Pus:** *R. pusillus* – 2 males (2,2%).

Data presented on Fig. 27, show the presence of all the 8 different morphological groups within the qualitative cluster 1. The inclusion of so many morphological groups, as distinct from each other such as *R. sanguineus* and *R. turanicus* for example; suggests that this is a qualitative cluster with large intra-specific variation, and therefore it is difficult to define the main morphological characteristics. Nevertheless it is possible to note that the less numerous morphological groups such as *R. sanguineus* D, *R. sanguineus R. turanicus* D and *R. pusillus* are represented by very few elements, morphological groups with an intermediate expression as *R. sanguineus s. s.* and *R. turanicus* have a reasonable expressiveness and the most expressive elements of sample *R. sanguineus* T1 and *R. sanguineus* T2 emerge as the dominant elements within this quantitative cluster. That works almost like a mirror of the total sample considered, once it presents all the morphological clusters of the sample, in identical proportions to the ones shown in the total sample.

Within the 122 specimens belonging to the qualitative cluster 2, 16 (13,1%) were classified as *R. sanguineus sensu stricto*, 43 (35,3%) as *R. sanguineus* Type 1, 40 (32,8%) were classified as *R. sanguineus* Type 2, eight (6,6%) as *R. sanguineus* D, three (2,5%) as *R. sanguineus* R and 12 (9,8%) as *R. turanicus* (Fig.28).

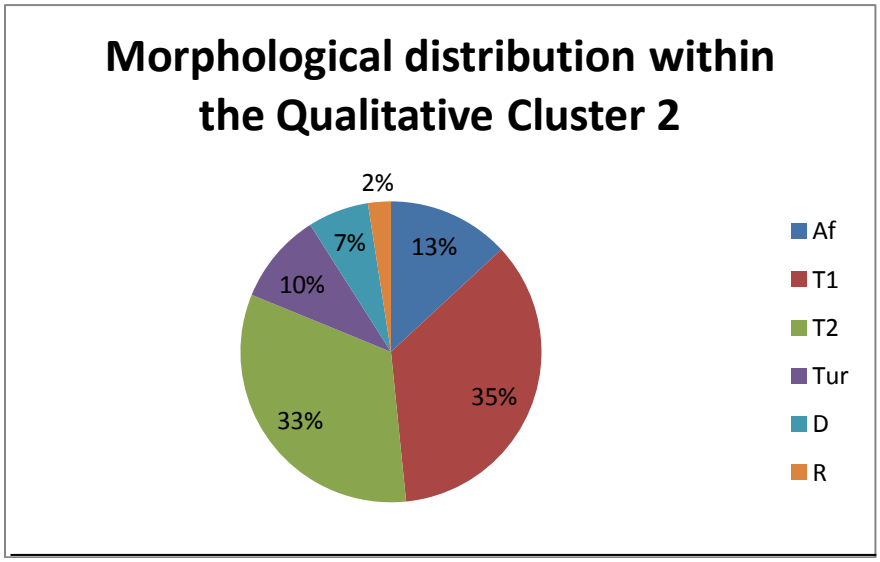


Fig.28: Morphologic distribution within the Qualitative Cluster 2- Graphic that relates the morphologic clusters obtained by morphologic analysis, with the qualitative cluster 1 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 16 males (13,1%); **T1:** *R. sanguineus* Type 1 – 43 males (35,3%); **T2:** *R sanguineus* type 2 – 40 males (32,8%); **D:** *R. sanguineus* D – 8 males (6,6%); **R:** *R. sanguineus* R – 3 males (2,5%); **Tur:** *R. turanicus* – 12 males (9,8%).

Data presented on Fig. 28, show the presence of six different morphological groups within the qualitative cluster 2. The presence of so many morphological groups, as distinct from each other; suggests that this is a qualitative cluster, with large intra-specific variation, and therefore it is difficult to define the main morphological characteristics. Nevertheless it is possible to note, that this is a similar situation to what was seen in the qualitative cluster 1, once the less numerous morphological groups such as, *R. sanguineus* R which have only a few individuals; morphological groups with an intermediate expression as *R. sanguineus s. s.* and *R. turanicus*, which have a reasonable expressiveness and the most expressive elements of sample *R. sanguineus* T1 and *R. sanguineus* T2, emerge as the dominant element. The main difference between this cluster and the qualitative cluster 1, is the fact there is a stronger presence of the morphologic cluster *R. sanguineus* D within the qualitative cluster 2.

The information regarding the qualitative cluster 3 shows that within the 34 specimens belonging to the qualitative cluster 3, three (8,8%) were classified as *R. sanguineus sensu*

stricto, 10 (30,3%) as *R. sanguineus* Type 1, seven (20,6%) as *R. sanguineus* Type 2, one (2,9%) as *R. sanguineus* D, three (8,8%) as *R. turanicus,* two (5,9%) as *R. turanicus* D and 8 (23,5 %) as *R. pusillus* (Fig.29).

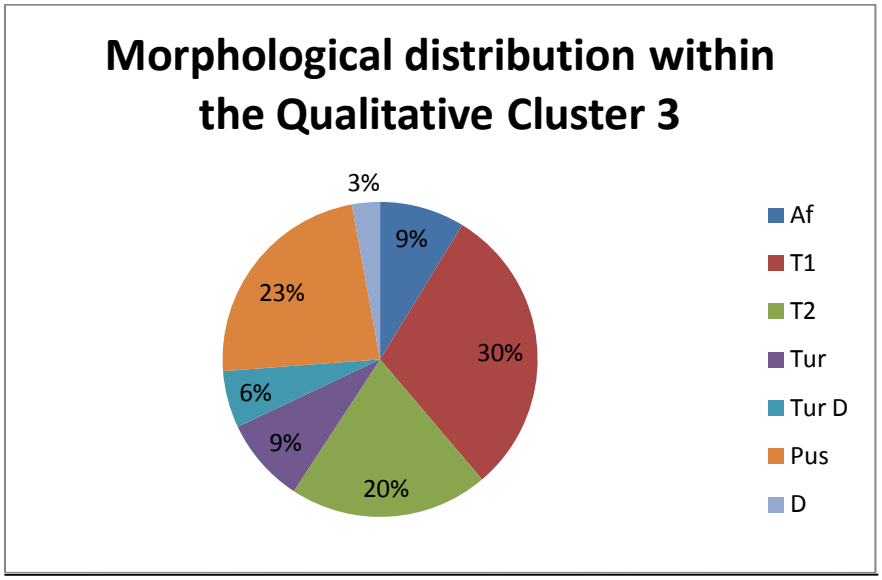


Fig. 29: Morphologic distribution within the Qualitative Cluster 3- Graphic that relates the morphologic clusters obtained by morphologic analysis, with the qualitative cluster 3 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 3 males (8,8%); **T1:** *R. sanguineus* Type 1 – 10 males (30,3%); **T2:** *R sanguineus* type 2 – 7 males (20,6%); **D:** *R. sanguineus* D – 1 males (2,9%); **Tur:** *R. turanicus* – 3 males (8,8%); **Tur D:** *R. turanicus D* – 2 males (5,9%); **Pus:** *R. pusillus* – 8 males (23,2%).

Data presented on Fig. 29, once again shows the presence of all the seven different morphological groups within the qualitative cluster 3. The presence of so many morphological groups, so distinct from each other suggests that this is once again a qualitative cluster with large intra-specific variation, and therefore it is difficult to define it´s main morphological characteristics. Nevertheless it is possible to evidence, some similaritiesto what was seen in the two previously analyzed quantitative clusters, once again, the less numerous morphological groups are *R. sanguineus* D and *R. turanicus D* represented only by a few elements; morphological groups with an intermediate expression as *R. sanguineus* and *R. turanicus* which have reasonable expressiveness; and are the most expressive elements of the sample *R. sanguineus* T1 and *R. sanguineus* T2, whom emerge as the dominant individuals. However this time these two morphologic clusters are followed by a third morphological cluster, with a very strong presence, *R. pusillus* which is the second most numerous morphologic group within this qualitative cluster. The fact that all specimens belonging *R. pusillus* species have been inserted into this cluster suggests that there is an association between the *R. pusillus* morphologic cluster and the quantitative cluster 3.

This association occurs because the species *R. pusillus* contains several morphological features, which differ from those of other morphological groups, described in this study, namely shorter dimensions, a distinctive spiracular area and their conscutums evidence a regular characteristic pattern of punctuation. Taking this into account, probably, the qualitative variables, "Spiracular area type", "Conscutum punctuation size" and "Conscutum punctuation distribution" were the ones that gave the bigger contribute to the insertion of *R. pusillus* specimens in the qualitative cluster 3.

4.2 Statistical and morphologic analysis - Females

4.2.1 Hierachical cluster analysis

Similar to what was done previously with males, Hierarchical Cluster Analysis was effectuated separately for quantitative and qualitative variables. This analysis was conducted in SPSS software [96] using the Ward method and the square of the Euclidian distance. These allowed obtaining the dendograms presented in Fig. 30 and Fig. 31.

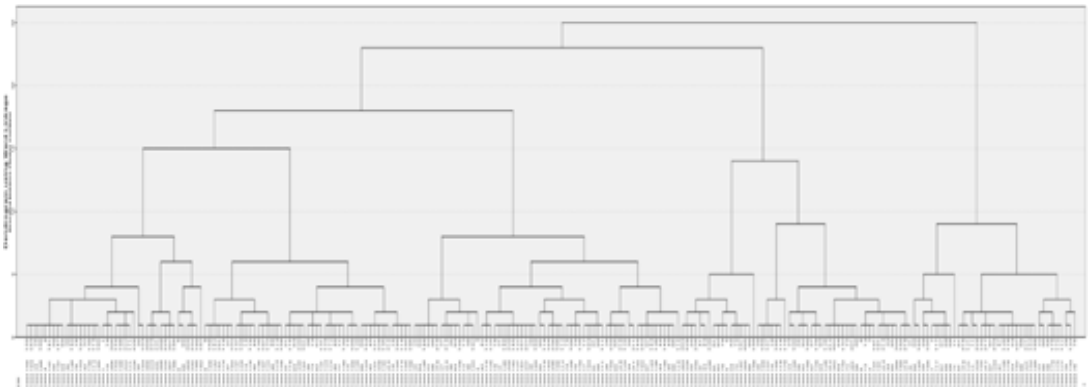


Fig. 30: Hierarchical Cluster Analysis dendrogram obtained with females quantitative variables data. The higher distance between fusions coefficients were obtained in the rescaled distance value 23 (forming 3 clusters).

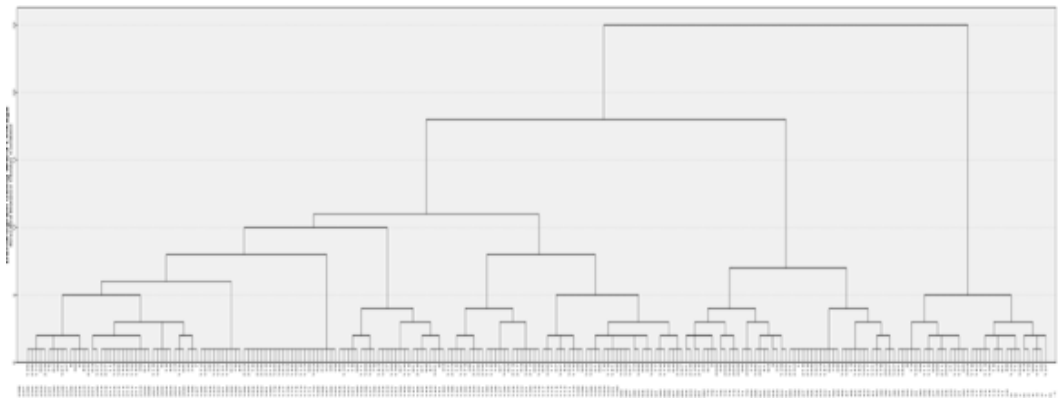


Fig. 31: Hierarchical Cluster Analysis dendrogram obtained with females' qualitative variables data. The higher distance between fusions coefficients were obtained in the rescaled distance value 18 (forming 3 clusters).

Just like previously described for males, the determination of the most appropriated number of clusters to be formed is presented in Table 6.

Table 6 - The last 10 fusion coefficients obtained in the Hierarchical Cluster Analysis.

Females					
Quantitative V.			**Qualitative V.**		
N. Clusters to form	**Fusion coefficient**	**S. of fusion coefficients**	**N. Clusters to form**	**Fusion coefficient**	**S. of fusion coefficients**
2	2115,00	172,394	2	111,589	13,080
3	1942,601	152,975	3	98,509	8,950
4	1789,625	118,472	4	89,559	5,604
5	1671,153	99,091	5	83,955	5,119
6	1572,062	94,841	6	78,836	3,859
7	1477,221	60,472	7	74,977	3,789
8	1416,749	55,822	8	71,188	3,225
9	1360,927	54,981	9	67,963	2,751
10	1305,946	39,208	10	65,211	2,498
11	1256,404	37,600	11	62,713	2,182
-	1217,196	-	-	60,531	-

Note: These values were used to determinate the number of clusters to form in the different statistical analysis performed. N- number, V. – variable, S.- Subtraction

In order to make these data easier to interpret, both quantitative and qualitative variables were represented using graphics and were presented in graphical form, in figure 32 and 33 respectively.

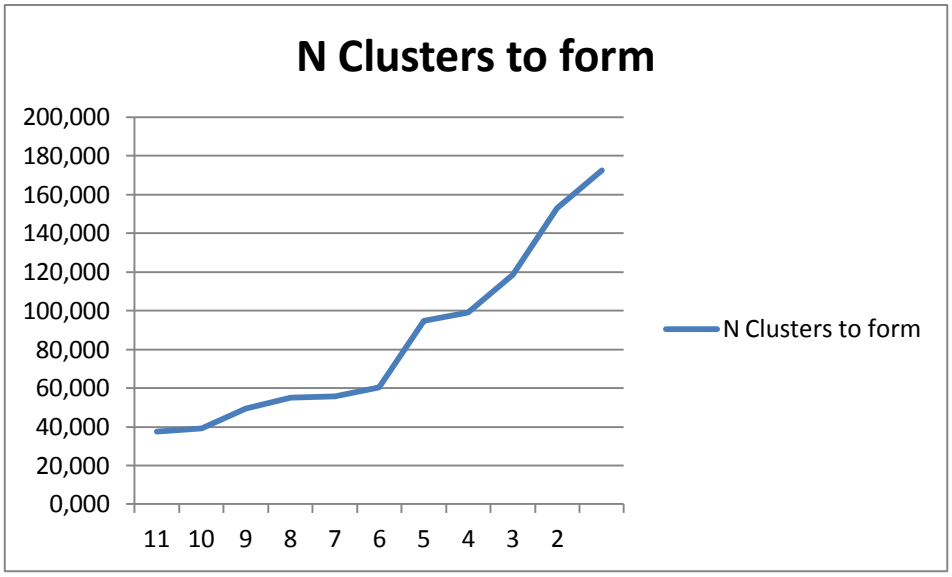


Fig. 32: Quantitative variables- the subtraction between the last 10 fusion coefficients, gave the differences between fusion coefficients'. The biggest differences are the ones that indicate the more appropriated number of clusters to form. In this case the biggest differences between fusion coefficients occur in position 3, followed by the position 6, therefore 3 our 6 clusters are both, adequate choices about the number of clusters to form. N- Number

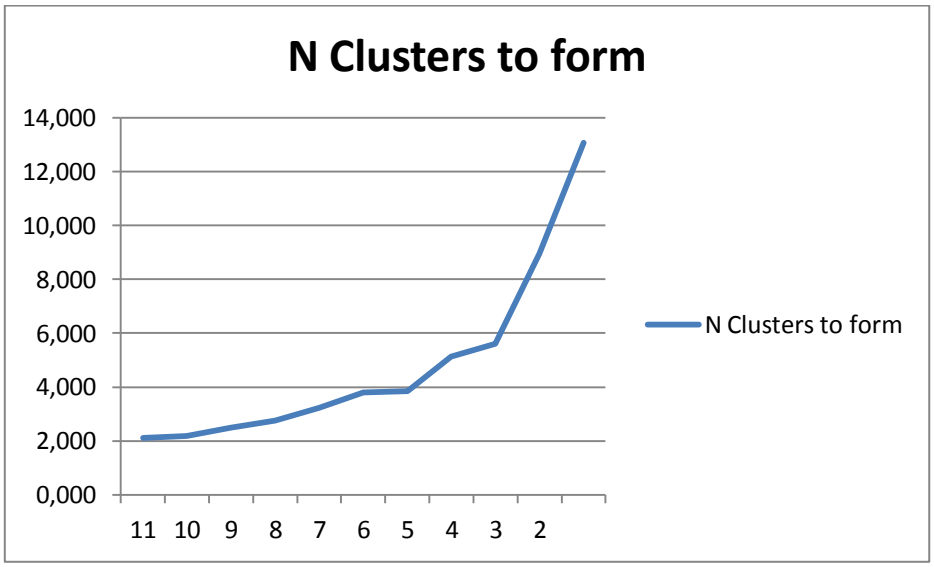


Fig. 33: Qualitative variables- the subtraction between the last 10 fusion coefficients, gave the differences between fusion coefficients. The biggest differences are the ones that indicate the more appropriate numbers of clusters to form. In this case the biggest difference occurs in position the 2, followed by the position 3, that are practically equivalent among themselves, therefore 2 and 3 clusters are both, adequate choices about the number of clusters to form. N- Number.

Considering the fusion coefficients distances obtained during the cluster analysis, the graphic display of those data, the information presented on the dendograms, and the nature of our sample it was decided to form three groups of clusters both for quantitative and qualitative variable analysis.

Therefore, in order to characterize the profile of these clusters, ANOVA statistical model was performed for quantitative variables and cross-tabulation statistics was effectuated for qualitative variables.

4.2.2 Quantitative clusters analysis

Attending to the objective of classifying the formed female's quantitative variables clusters characteristics; a one-way ANOVA statistical analysis was performed. This analysis allowed characterizing the morphological features of our female sample in relation to several descriptive measures, measures that are displayed in table 7.

Table 7 – Females descriptive statistics of quantitative variables within the clusters formed by hierarchical cluster analysis. All measures were taken in millimeters, except the "Spiracular areas tail angle" taken in angle degrees. N- Number of elements within the clusters, Std. Deviation – standard deviation, Std. Error – standard error.

Morfological Feature	Cluster	Descritive measures					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Scutum lenght/width ratio	1	147	0,972	0,106	0,009	0,682	1,440
	2	52	0,889	0,119	0,016	0,666	1,131
	3	37	0,879	0,183	0,030	0,589	1,079
	Total	236	0,939	0,130	0,009	0,589	1,440
Basis capituli height/width ratio	1	147	0,860	0,044	0,004	0,682	0,973
	2	52	0,854	0,081	0,011	0,650	1,146
	3	37	0,838	0,058	0,010	0,724	0,960
	Total	236	0,855	0,057	0,004	0,650	1,146
Porose areas height/width ratio	1	147	1,149	0,134	0,011	0,950	1,695
	2	52	1,106	0,099	0,014	0,859	1,396
	3	37	1,099	0,082	0,014	1,000	1,341
	Total	236	1,143	0,122	0,008	0,859	1,695
Spiracle oval area height/width ratio	1	147	0,651	0,143	0,009	0,351	0,902
	2	52	0,710	0,092	0,013	0,505	0,924
	3	37	0,648	0,082	0,013	0,465	0,885
	Total	236	0,664	0,107	0,007	0,352	0,921
Spiracle tail length/width differentiation	1	147	1,658	0,533	0,044	0,571	2,971
	2	52	1,297	0,281	0,039	0,766	1,952
	3	37	1,785	0,526	0,086	0,558	2,953
	Total	236	1,598	0,544	0,033	0,558	2,971

Table7– (Continued)

Morphological Feature	Cluster	Descriptive measures					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Spiracle area angle	1	147	90,454	12,400	1,023	53,034	130,996
	2	52	81,766	10,272	1,424	64,355	103,724
	3	37	95,153	11,884	1,954	68,618	117,075
	Total	236	89,276	12,604	0,820	53,034	130,996
Sclerites width/length ratio	1	147	0,549	0,097	0,008	0,285	0,852
	2	52	0,601	0,142	0,020	0,370	0,886
	3	37	0,682	0,090	0,015	0,502	0,873
	Total	236	0,581	0,117	0,008	0,285	0,886
Sclerites insertion ratio	1	147	0,674	0,229	0,019	0,098	1,324
	2	52	0,530	0,178	0,025	0,205	0,926
	3	37	1,235	0,305	0,050	0,827	2,192
	Total	236	0,730	0,323	0,021	0,098	2,192
Genital pore aperture	1	147	74,657	11,515	0,950	53,034	130,996
	2	52	52,138	21,137	2,931	64,353	103,724
	3	37	71,138	12,131	1,994	68,618	117,075
	Total	236	69,190	16,903	1,100	53,034	130,996

Note: The variable "Spiracular tail length/width differentiation" consists in a ratio between the length of tail of the spiracle and the difference between the width of tail basis of the spiracle tail and the final width of the spiracle tail.

The descriptive statistic measurement used to explain these three clusters was the mean. The means of the quantitative variables within the 3 clusters formed are graphically displayed in Fig. 34, Fig. 35 and Fig. 36. The "Spiracular areas tail angle" graphic is apart from the others, by the same reasons presented in the male's analysis. In females, however there is another quantitative variable, "Genital pore aperture" whose graph is apart from the others, because although it presents the same unit of measurement as the others variables "millimeters", once it was not treated through a ratio, it shows values with different dimension.

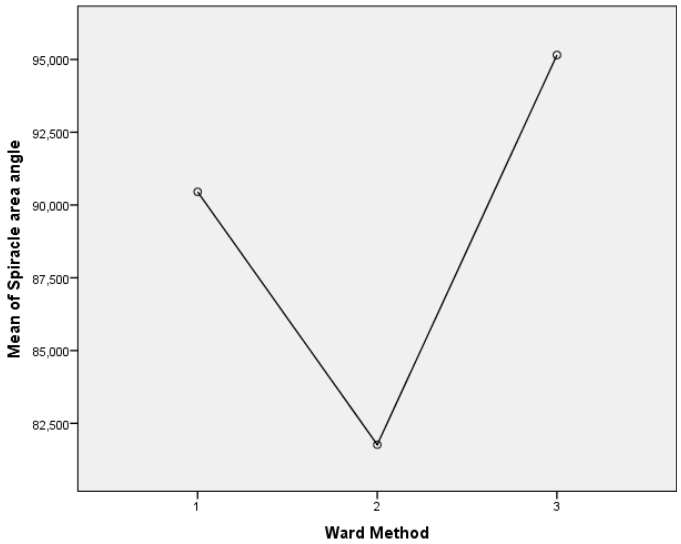


Fig. 34: "Spiracle area angle" quantitative variable female's clusters means. In comparison to the others quantitative variables, this one presents the highest mean values; this is a result of this variable being measured in angles not millimeters. When comparing the 3 clusters among themselves, we can observe that the mean clearly defined the different clusters; 2-3 are particularly well differentiated. The Cluster means are Cluster 1 – μ=90,454, Cluster 2 – μ=81,765 and Cluster 3 – μ=95,153.

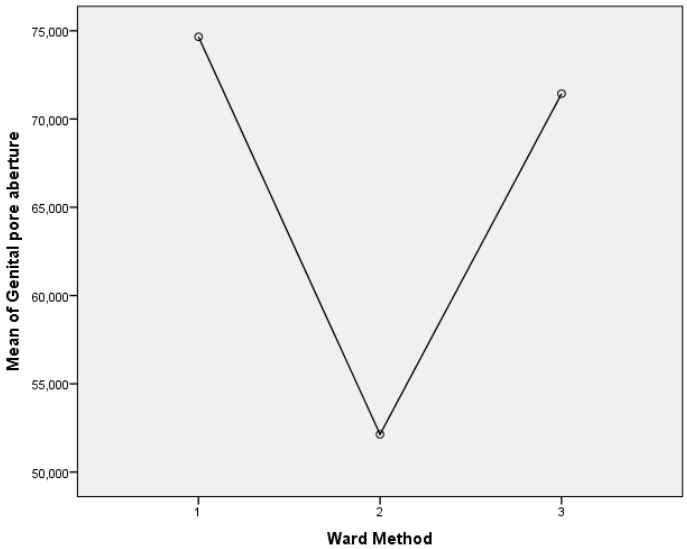


Fig. 35: "**Genital pore aberture" quantitative variable female's clusters means.** In comparison to the others quantitative variables, measured in millimeters this one presents the highest values; this is result of this variable not having been treated, with a ratio, when comparing the 3 clusters among themselves, the one that is better differentiated by its mean is cluster 2, in turn the clusters 1 and 3 are poorly differentiated, since they have very similar means. The Cluster means are Cluster 1 – μ=74,656, Cluster 2 – μ=52,137 and Cluster 3 – μ=71,438.

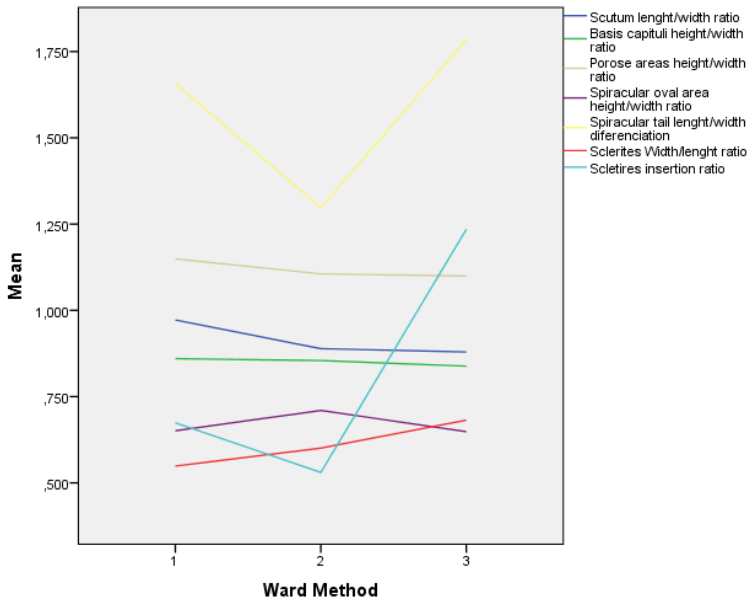


Fig. 36: Clusters means obtained based on all females quantitative variables less the spiracular angle and the genital pore aperture. The variables where the means plainly defines the different clusters were "Sclerites width/length ratio" and "Sclerites insertion ratio". The following variables failed to differentiate the means of two clusters "Porose height/width ratio" of the 2-3, "Spiracular oval area height/width ratio" of the 1-3 "Spiracle tail length/width differentiation" of the 1-3, "Scutum length/width ratio" of the 2-3, "Basis capituli height/width ratio" of the 1-2.

Considering data displayed on table 7 and figs. 34, 35 and 36, the following conclusions regarding the contribution of the quantitative variables means to the differentiation between clusters, can be presented:

- The variables, where the means clearly defined the distinct clusters were "Sclerites width/length ratio", "Sclerites insertion ratio" and "Spiracle area angle".

- The variables that failed to differentiate the means of two clusters were "Porose areas height/width ratio", "Spiracular oval area height/width ratio" and "Spiracle tail width/height differentiation", "Scutum length/width ratio", "Basis capituli height/width ratio" and "Genital pore aperture" these variables did not differentiated the means of the 2-3, 1-3, 1-3, 2-3, 1-2 and 1-3 clusters, respectively.

Considering the results above it is possible to characterize the three distinct clusters as follows:

- The cluster 1 contains specimens with the largest scutums of the 3 clusters formed, it also presents large capitulum, with average size porose areas; the ticks within this cluster also have large spiracles, with long tails, with average width, and medium angles;

simultaneously, the elements within this cluster also exhibit the highest sclerites and the widest genital aperture pore of the sample.

- The cluster 2 presents specimens with the smallest scutums and also the smallest capitulum, this cluster also presents medium sized porose areas; average size spiracles, whose display tails that have short lengths, and narrow widths; and evidence the smallest angles. The specimens within this cluster also display the narroest genital pore aperture and sclerites with slightly under average dimensions.

- The cluster 3 includes individuals that own medium sized scutums, large capitulum, porose areas with average dimensions. The specimens within this cluster also show large spiracles with the largest angles of the 3 clusters formed and tall and thin spiracular tails; and also, a wide genital pore aperture, and sclerites with short lengths and large widths.

As it had previously been done for males, with the purpose of classify the statistical significance of the obtained results, an ANOVA statistical analysis was effectuated. The following values were acquired:

- The results of the variable “Basis capituli height/width ratio” were p=0,119 and F=2,153. This indicates that the variable did not significantly differentiate the means between clusters.

- The “Sclerites insertion ratio” variable results were p=0,000 and F=110,520, the variable “Genital pore aperture” presented the results; p=0,000 and F= 48,377, the “Sclerites width/length ratio” variable results were p=0,000 and F= 23,778, the results of the variable “Spiracle area angle” were p=0,000 and F= 15,627, the variable “Scutum length/width ratio” presented the results p=0,000 and F= 13,873, the “Spiracular tail length/width differentiation” variable results were p=0,000 and F=13,694, the variable “Spiracular area width/height ratio” results were p=0,002 and F=6,549, finally the variable“ Porose areas height/width ratio” presented the results p= 0,019 e F= 4,051.

- These results indicate that, except for the variable "Basis capitulli height/width ratio", all the others quantitative variables considered were statistical significantly differentiated by the clusters means, suggesting that all of them gave a significant contribute for the clusters

formation. Considering the F-value, the variables are presented in descending order of significance for the cluster formation, meaning the variable that more significantly contributed for the clusters formation was the “Sclerites insertion ratio” and the variable that less significantly contributed for the cluster formation was the “Porose areas height/width ratio”.

Usually used in conjunction with ANOVA, a multiple comparison Tukey HSD test (post hoc test) was performed. It is a single-step multiple comparison procedure, that works as a statistical tool, permiting to find the means that are significantly different from each other. This test allowed obtaining the following results:

- The “Basis capituli height/width ratio” variable presented results that did not achieve the condition of the test: H0 hypothesis ($p \leq 0{,}050$), as consequence of this, it cannot be evaluated, and it is not statistically significant for the differentiation of the clusters means.

- The variable “Porose areas height/width ratio” failed to present a statistically significant difference between all 3 clusters, 1-2, 2-3 and 1-3 ($p=0{,}069$, $p=0{,}067$ and $p=0{,}967$) respectively.

- Despite some statistically significant p-values were observed; the “Scutum length/width ratio” variable did not present a statistically significant difference between the 2-3 cluster means ($p=0{,}931$); the variable “Spiracular oval area height/width ratio” did not show a statistically significant difference between the 1-3 clusters means ($p=0{,}967$); the “Spiracular tail length/width differentiation” variable did not evidence a statistically significant difference between 1-3 clusters means ($p=0{,}335$); the “Spiracle area angle” variable did not displaysed a statistically difference between the 1-3 clusters means ($p=0{,}082$); the variable “Genital pore aperture” did not demonstratesa statistically significant difference between 1-3 clusters mean ($p=0{,}439$).

- The variables “Sclerites width/length ratio” and “Sclerites insertion ratio” are the ones that gave the highest contribute to the clusters formation, because these variables are both able to statistically differentiate with significance all the clusters means, once they both exhibit exclusively $p<0{,}050$ between the 3 clusters.

4.2.3 Qualitative Variable clusters analysis

In order to classify the formed females' qualitative variables clusters, a cross-tabulation statistics was performed from the 236 females present on this study. The females' qualitative variables clusters characterization by percentage is described for each of the 3 clusters that information is presented in annexes in pages 146 and 147.

The results relative to the association measure Cramer´s V and Chi-square test were acquired for all variables relatively to the qualitative variables groups, and are presented as follows:

- The variables "Cervical fields depression", "Cervical grooves definition", "Capituli palp shape 2ndA" and failed to meet the test condition (<20% of cells with expected count less than 5 and minimum expected counts higher that 1) therefore they will not be interpreted.

- The "Genital aperture form" variable results were p=0,000, (χ2(1)=224,330 and V=0,687; indicating that this variable has a statistically significant strong relationship and as it shows the highest V-value, meaning it is the main variable for the qualitative variables groups formation.

- The variable "Scutum posterior margin" evidenced the results p=0,000, (χ2(1)= 143,286 and V=0,551, therefore it has a moderate statistically significant effect on the qualitative clusters formation.

- The "Scutum punctuation size" variable results were: p=0,000 (χ2(1)=24,238 and V=0,227, the "Cervical fields shape" variable results were: p= 0,000 (χ2(1)=23,350 and V=0,222, and the "Scutum punctuation distribution" results were: p=0,007 (χ2(1)=14,019 and V=0,172, meaning they have a low statistical significant effect on the qualitative clusters formation. Considering the V-value these variables are in descending other of contribution for the qualitative clusters formation.

- The "Cervical fields setiferous punctuations size" variable results were p=0,587 χ2(1)=1,066 and V=0,067; which indicate that this variables do not have a statistical significant effect on the qualitative clusters formation.

If we look at the results obtained so far, it is possible to mention that the quantitative variables that gave the most contribute to the quantitative cluster formation were “Sclerites insertion ratio” and “Sclerites height/width ratio” followed by the variables “Genital pore aberture”, “Spiracle area angle”, “Spiracular oval area length/width differentiation”, “Spiracular oval height/width ratio” and “Basis capituli length/width ratio”. Among the variables that statistically contributed for the quantitative clusters formation “Porose areas height/width ratio” was the one that less contributed. The results also indicate that the variable “Basis capituli height/width ratio” did not contributed for the quantitative clusters formation

Considerading the qualitative clusters formation the variable that had the most statistically significant effect on the qualitative clusters formation was “Genital aperture form”. “Scutum posterior margin” has a moderate statistical significant effect. Results also indicate that the variables “Scutum punctuation size”, “Cervical fields shape”and “Scutum punctuation distribution” contribute for the qualitative clusters formation with a weak statistical significant effect on cluster formation. Finally all the other qualitative variables do not have a statistical significant effect on the quantitative clusters formation.

4.2.4 Correspondence analysis

Just like the analysis that was performed on the male specimens, the data presented on female study contains both quantitative and qualitative variables that lead to the formation of two types of clusters. Concerning the associations between the quantitative and qualitative clusters, a correspondence analysis was performed on the quantitative and qualitative variables clusters previously formed.

The obtaining results with the inertia value=0,084, are adequate, if we consider that a total above 0,20 must be achieved in other to acquire proper representations, and chi-square test, ($p=0,000$ and $\chi2(1)=22,110$). These results suggest the presence of a significant statistical strong correlation between both variables. As a consequence the output showed in fig. 37 reveals associations between the clusters 1 and 2 of the quantitative and qualitative typology respectively; the association between the cluster 2 of the quantitative typology and the cluster 3 of the qualitative typology; association between the cluster 1 of the qualitative typology and the cluster 3 of the quantitative typology; and the association between the cluster 2 and 1 of the quantitative and qualitative typology, respectively.

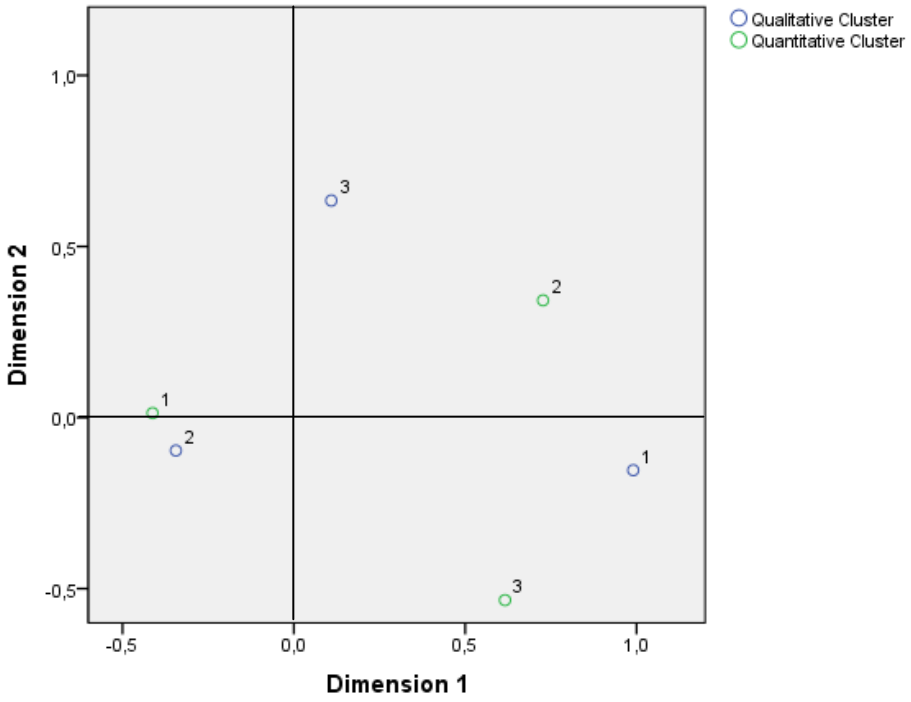


Fig. 37: Bivariate graph obtained from correspondence analysis of the females´ qualitative variables with the quantitative variables formed clusters: The results I=0,085 and p=0,001 suggest the presence of a statistical significant correlation, between both types of variables considered, the data presented on this bivariate graph allow to evidence association between the clusters 2-1, 1-3, 3-2 and 1-2 of the variables qualitative and quantitative, respectively.

4.2.5 Morphologic Classification

Alongside with the hierarchical cluster analysis effectuated for quantitative and qualitative variables, conducted in SSPS software [96] a morphologic analysis was performed in all 236 females contained in our sample.

Using this method and these criteria, our female sample contained specimens that belong to the following morphologic groups: *R. sanguineus sensu stricto* (Africanus), *R. sanguineus* T1, *R. sanguineus* T2, *R. turanicus*, *R. pusillus*, and also one other morphological group that have not been described before, it was decided to name it: "*R. sanguineus* Intermediate"

Among this sample, only the females exhibiting characteristics of *R. sanguineus s. s.* such as, spiracles with tall and narrow tails, wide genital aperture presenting the pattern 2, were classified as such, the same was applied for *R. turanicus*, only females with characteristics of the species *R. turanicus*, especially spiracles, with short and wide tails and narrow genital opening exhibiting the pattern 5, were distinguished as such. These criteria, allowed many of this sample being classified as intermediate forms, such as *R. sanguineus* T2 but and *R. sanguineus* T1.

As so, *R. sanguineus* Type 1 refers to intermediate forms which have in common more characteristics with *R. sanguineus s. s.* than with *R. turanicus*, namely they both share pattern 2 wide genital apertures. The difference between these specimens and those classified as *R. sanguineus* Type 2 is that, this typology shares less characteristics with *R. sanguineus*, for example: they present spiracles with shorter, wider tails, and pattern 3 genital apertures, diverging more from *R. sanguineus* than the type 1 typology.

The *R. sanguineus* Intermediate morphologic group refers to ticks that display spiracles with an intermediate form between what is described as *R. sanguineus s. s.* and *R. sanguineus T2*, typologies. However, it is also something different from what is seen in *R. sanguineus* T1 as its spiracles bodies aren't so globular. In addition, this group exhibits a pattern 4 genital aperture. Our sample also displays some specimens that belong to the species *R. pusillus*, which were included in this study as an outlier, or control-group.
Note: All the 6 morphologic groups mentioned in this paragraph are, detailed described, with text and images in pages 84 to 87.

The six morphologic groups observed in this sample can be converted in morphologic clusters, in order to be analyzed alongside with the qualitative and quantitative cluster previously grouped, as it had been done for the male sample.

The information of each female element regarding the taxonomic group to which it belongs as well as the quanlitative and quantitative clusters, where it was previously inserted alongside with collection region and the identifying number of each specimen is displayed in table 8 presented in appendices page 153.

By taking a closer look, at the information displayed at table 8, it is concluded that: in the 236 females considered for this study, 54 (22,9%) specimens were collected in Setúbal, 12 (5,1%) in Alcochete, six (2,5%) in Peniche, 51 (21,6%) in Alcobaça and 113 (47,9%) in Vila Franca de Xira (fig. 38). In this study, the geographic distribution of the specimens will not be considered due to the low number of specimens collected specially in Peniche and Alcochete.

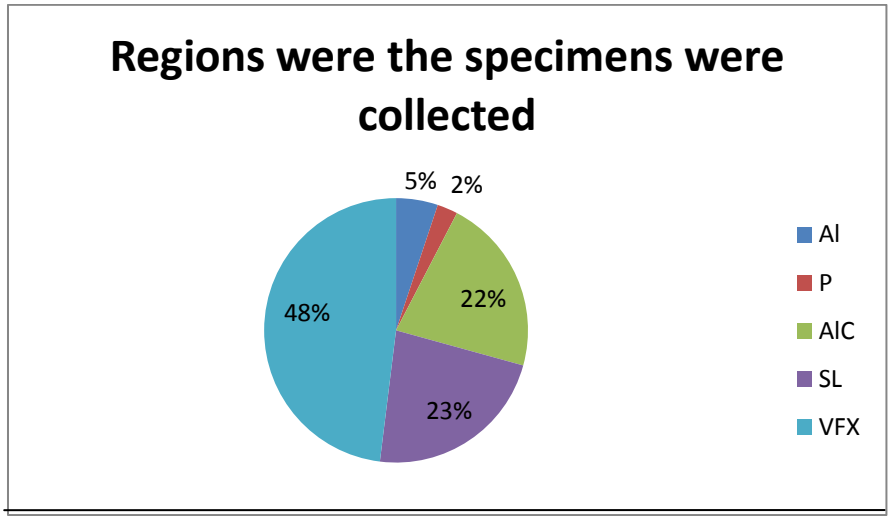


Fig. 38: Regions were the specimens were collected- Graphic summarizing the information relative to the amount of male specimens collected in each region: **Sl:** Setúbal –54 females (22,9%); **Al:** Alcochete – 12 females (5,1%); **P:** Peniche – 6 females (2,5%); **ALC:** Alcobaça – 51 females (21,6%); **VFX:** Vila Franca de Xira – 113 females (47,9%).

Table 8 also provides information about the morphological cluster where each specimen is included. Analyzing the sample based on this parameter it is possible to state that: in the 236 females, 39 (16,5%) were classified as *R. sanguineus sensu stricto*, 28 (11,9%) as *R. sanguineus* type 1, 116 (49,1%) as *R. sanguineus* type 2, 21 (8,8%) as *R. sanguineus* Intermediate, 12 (5,1%) as *R. turanicus* and 20 (8,5) as *R. pusillus* (fig. 39).

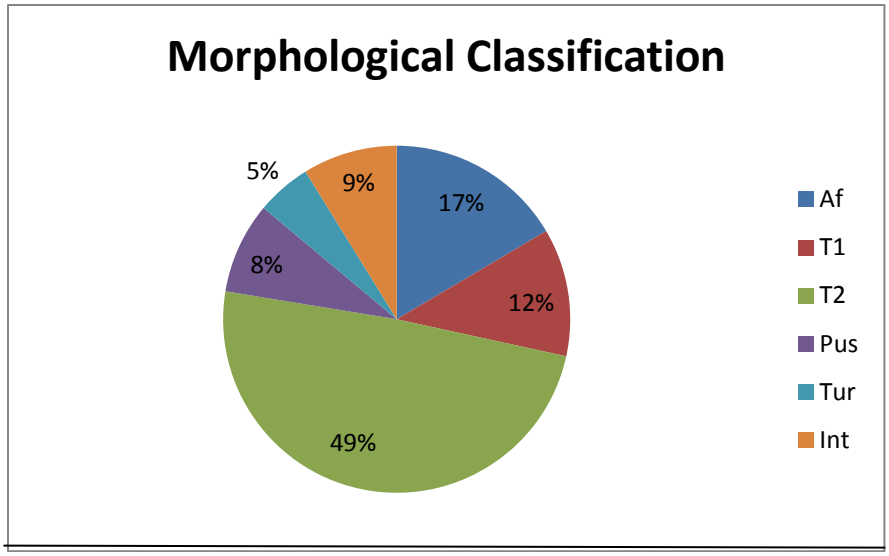


Fig. 39: Morphologic Classification- Graphic that synthesizes the information, relative to the morphological classification within the sample considered in this study**: Af:** *R. sanguineus sensu stricto* – 39 females (16,5%); **T1:** *R. sanguineus* Type 1 – 28 females (11,9%); **T2:** *R sanguineus* type 2 – 116 females (49,1%); **Int:** *R. sanguineus* Int – 21 females (8,8%); **Tur:** *R. turanicus* – 12 females (5,1%); **Pus:** *R. pusillus* – 20 females (8,5%).

Alongside with the data on the morphological cluster that each specimen belongs, table 8 also displays information concerning the quantitative and qualitative clusters where each female is included, therefore it is possible to relate morphological clusters to quantitative and qualitative clusters, respectively.

In order to understand what are the morphological groups, appearing more frequently associated with each of the quantitative clusters and also, how this relationship occurs in the quantitative clusters, it is necessary to describe morphological characteristics associated to each morphological clusters which is exposed in Table 9.

Table 9 – Females descriptive statistics of quantitative variables within the morphologic clusters formed by.

Morphological Feature	Morphological Cluster	Descriptive measures					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Scutum lenght/width ratio	**T1**	28	,881	,132	,025	,589	1,069
	T2	116	,942	,130	,012	,088	1,440
	Af	39	,952	,122	,019	,652	1,125
	Int	21	,983	,145	,031	,696	1,335
	Tur	12	,966	,113	,032	,730	1,131
	Pus	20	,920	,118	,026	,712	1,077
	Total	236	,939	,130	,008	,088	1,440
Basis capituli height/width ratio	**T1**	28	,831	0,049	,009	,749	,931
	T2	116	,856	,050	,005	,702	,973
	Af	39	,846	,052	,008	,682	,938
	Int	21	,874	,078	,017	,779	1,146
	Tur	12	,869	,092	,027	,650	1,057
	Pus	20	,875	,050	,011	,749	,957
	Total	236	,856	,057	,004	,650	1,146
Porose areas height/width ratio	**T1**	28	1,103	,078	,015	1,000	1,284
	T2	116	1,13	,125	,012	,859	1,695
	Af	39	1,166	,156	,025	1,000	1,578
	Int	21	1,100	,108	,024	,950	1,396
	Tur	12	1,137	,095	,028	1,000	1,308
	Pus	20	1,108	,091	,020	1,000	1,300
	Total	236	1,132	,121	,008	,859	1,695
Spiracle oval area height/width ratio	**T1**	28	,696	,103	,019	,506	,921
	T2	116	,662	,090	,008	,457	,887
	Af	39	,658	,109	,017	,352	,902
	Int	21	,659	,109	,024	,505	,860
	Tur	12	,672	,060	,017	,582	,764
	Pus	20	,671	,119	,026	,441	,855
	Total	236	,667	,097	,006	,352	,921
Spiracle tail lenght/width differentiation	**T1**	28	1,55	,395	,075	,558	2,444
	T2	116	1,64	,504	,047	,571	2,953
	Af	39	1,60	,528	,085	,608	2,872
	Int	21	1,96	,636	,139	,875	2,971
	Tur	12	1,39	,389	,112	,867	1,952
	Pus	20	1,20	,293	,066	,766	1,800
	Total	236	1,60	,514	,033	,558	2,971

Table 9 – (Continued)

Morphologic Feature	Morphologic Cluster	Descriptive measures					
		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Spiracular area angle	**T1**	28	90,620	13,531	2,557	68,618	117,075
	T2	116	90,124	12,595	1,169	65,159	130,996
	Af	39	91,011	12,345	1,976	53,034	116,532
	Int	21	91,014	9,267	2,022	69,444	108,269
	Tur	12	83,798	13,740	3,966	64,353	103,393
	Pus	20	80,558	11,151	2,493	66,184	104,503
	Total	236	89,276	12,603	,820	53,034	130,996
Sclerites Width/lenght ratio	**T1**	28	,673	,093	,018	,483	,833
	T2	116	,531	,090	,008	,285	,748
	Af	39	,657	,112	,018	,452	,886
	Int	21	,565	,106	,023	,415	,759
	Tur	12	,475	,0670	,019	,370	,597
	Pus	20	,670	,122	,027	,459	,874
	Total	236	,581	,117	,007	,285	,886
Scletires insertion ratio	**T1**	28	,997	,376	,071	,229	1,921
	T2	116	,766	,309	,029	,171	2,192
	Af	39	,724	,252	,040	,210	1,649
	Int	21	,512	,243	,053	,098	1,213
	Tur	12	,445	,165	,047	,205	,812
	Pus	20	,560	,250	,055	,236	1,228
	Total	236	,730	,323	,021	,098	2,192
Genital pore aperture	**T1**	28	73,475	13,3600	2,524	45,281	100,111
	T2	116	75,404	10,285	,954	50,139	108,095
	Af	39	73,032	11,047	1,768	49,183	97,943
	Int	21	71,580	9,905	2,161	54,845	89,669
	Tur	12	24,569	4,360	1,258	19,400	32,346
	Pus	20	43,920	7,246	1,620	32,202	61,748
	Total	236	69,190	16,903	1,100	19,400	108,095

Note: All measures were taken in millimeters, except the Spiracle area angle taken in angle degrees. N- Number of elements within the clusters, Std. Deviation – standard deviation, Std. Error – standard error.

Considering the results above the following characterization for the 6 distinct morphologic clusters are:

- The *R. sanguineus* T1 morphologic cluster presents elements with medium sized scutums, average capitulum, with medium sized porose areas, spiracles with average dimensions that display thin tails with medium lengths and average angle. This cluster also includes specimens which genitalia exibitis medium sized sclerites with short lengths and a wide genital aperture displaying the pattern 2.

- The *R. sanguineus* T2 morphologic cluster contains individuals with large scutums, large capitulum, with medium sized porose areas, large spiracles, with high and medium widths tails and the large angles. This cluster also comprises specimens with high and wide sclerites and a wide pattern 3 genital aperture.

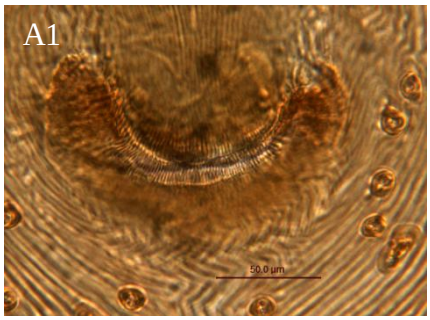
- *R. sanguineus* s. s. (Af) morphologic cluster displays specimens with large scutums, large capitulum with average size porose areas, large spiracles with large angles and with the narrowest and highest tails of the sample. This cluster also shows high and wide sclerites and a pattern 2 wide genital aperture.

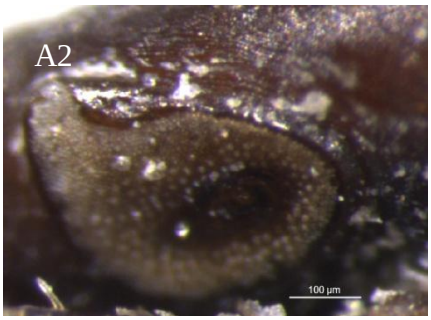
- *R. sanguineus* Int morphologic cluster includes elements with the largest scutums, and also the largest capitulum of the sample, large spiracles with large angles and thin tails with average height. This cluster also displays high and wide sclerites and a pattern 4 wide genital aperture.

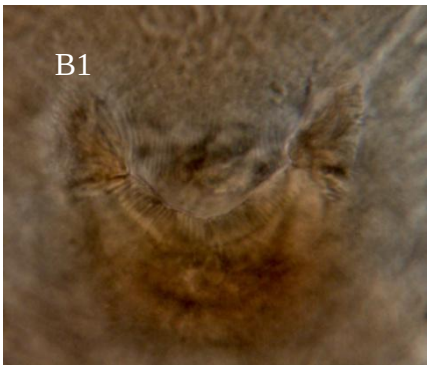
- *R. turanicus* morphologic cluster contains mostly specimens with large scutums, medium sized capitulum and the largest spiracles of the 6 morphologic clusters formed, exhibiting small angles and short tails with large widths. This cluster also displays the sclerites with the greatest heights of the sample and narrow pattern 5 genital aperture.

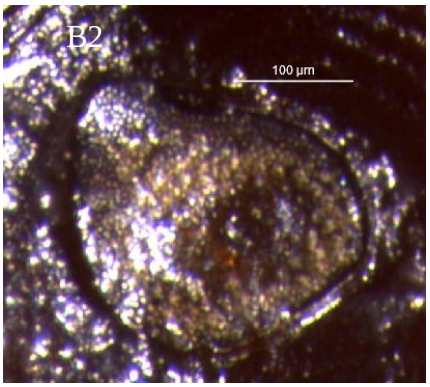
- *R. pusillus* morphologic cluster presents individuals with the smallest scutums, the smallest capitulum, among the population considered for this study. This cluster also displays the shortest spiracles of the sample, with the lowest tail angles of the 6 clusters formed, short and wide spiracular tail. These ticks also show the lower scletites of all the taxonomic clusters, and the second narrowest genital aperture, pattern 1.

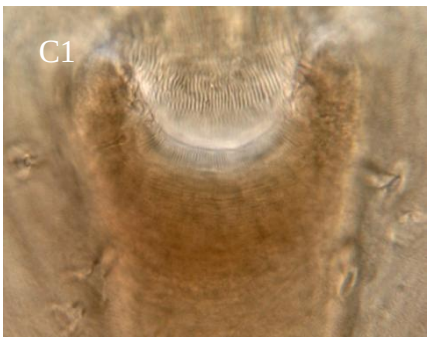
It is possible to conclude then, when it cames to females, the main morphologic differences between the six morphologic clusters formed are related to the different structures presented by the genital aperture, and by the spiracular area, this differences are evidenced in fig 40.

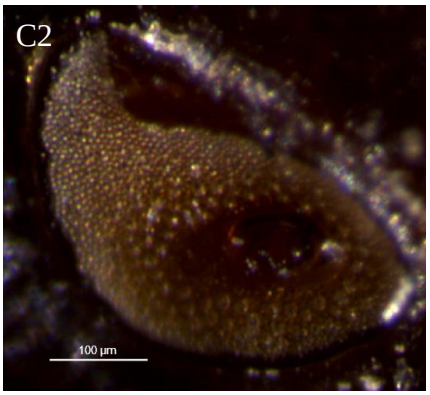

A1
50.0 µm

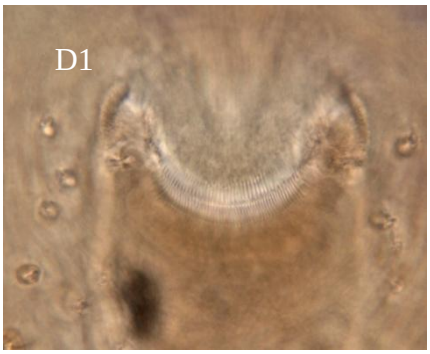

A2
100 µm

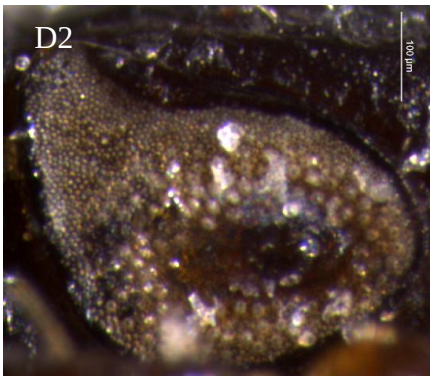

B1


B2
100 µm


C1


C2
100 µm


D1


D2
100 µm

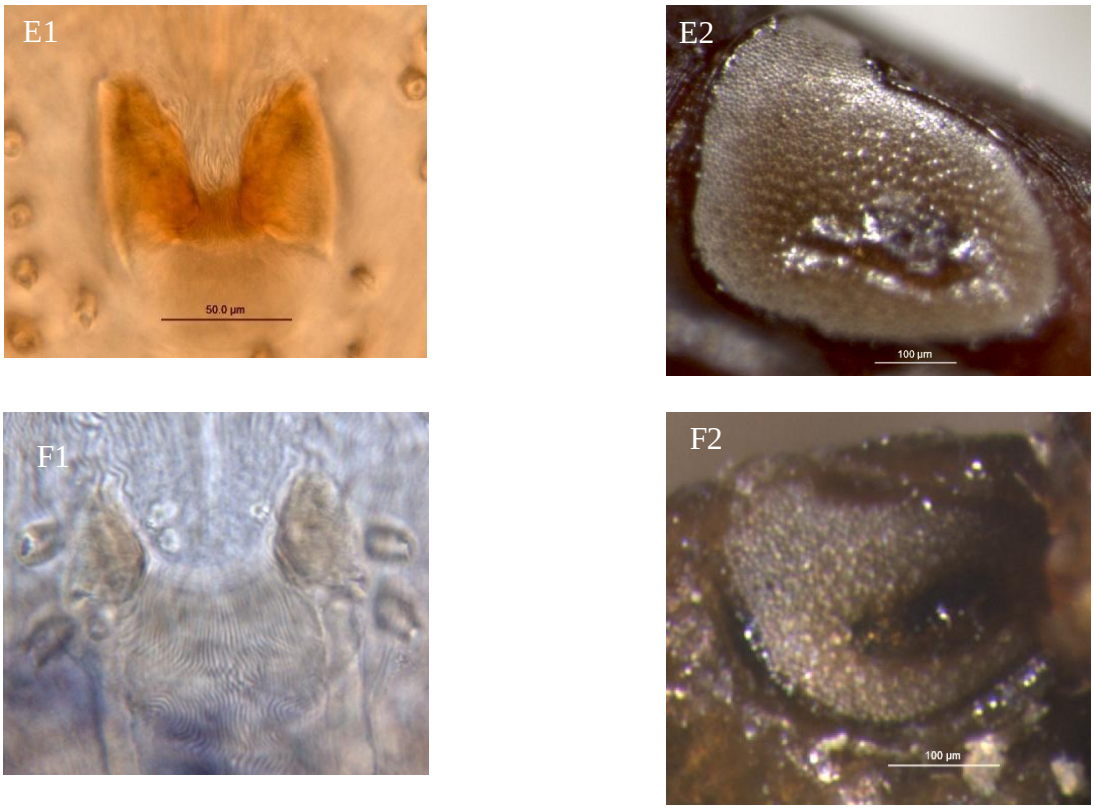


Fig. 40: Main distinctive features of females: A - *Rhipicephalus sanguineus* s. s. (africanus), A1- Pattern 2 genital aperture, A2 – Spiracular plate; B - *R. sanguineus* type I; B1 - Pattern 2 genital aperture, B2 - Spiracular plate; C- *R. sanguineus* type II, C1 – Pattern 3 genital aperture, C2 - Spiracular plate; D - *R. sanguineus* Int, D1- Pattern 4 genital aperture, D2 - Spiracular plate ; E - *R. turanicus*, E1 - Pattern 5 genital aperture, E2 – Spiracular plate; F – *R. pusillus*, F1 – Pattern 1 genital aperture, F2- Spiracular plate.

By analyzing individually each quantitative cluster, it is possible to observe that within the 147 specimens belonging to the quantitative cluster 1, 28 (19,0%) were classified as *R. sanguineus s. s.*, three (2,0%)as *R. sanguineus* Type 1, 93 (63,2%) as *R. sanguineus* Type 2, 17 (11,6%) as *R. sanguineus* Intermediate and six (4,0%) as *R. pusillus* (fig 41).

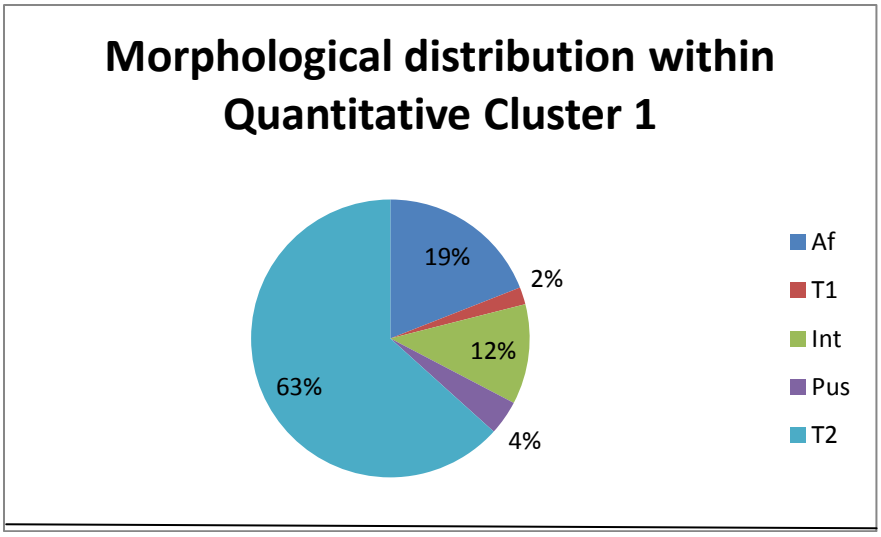


Fig. 41: Morphologic distribution within the Quantitative Cluster 1- Graphic that relates the taxonomic clusters obtained by morphologic analysis, with the quantitative cluster 1 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 28 females (19,0%); **T1:** *R. sanguineus* Type 1 – 3 females (2,0%); **T2:** *R sanguineus* type 2 – 93 females (63,2%); **Int:** *R. sanguineus* Int – 17 females (11,6,%); **Pus:** *R. pusillus* – 6 females (4,0%).

Data on Fig. 41, suggest five morphological groups in this cluster and within this five, it's easy to conclude that *R. sanguineus* T2 clearly dominates over the others, so it is possible to establish an association between this morphologic cluster and quantitative cluster 1.

However, despite this association being the strongest evidenced, by analyzing the constitution of this quantitative clusters more closely, it is possible to note the presence 17 specimens that were classified as *R. sanguineus* Intermediate represent 81% of the total sample of the *R. sanguineus* Intermediate morphologic cluster. The same situation occurs with 28 individuals that were classified *R. sanguineus* s. s. correspond to 72% of the total sample of the morphologic cluster *R. sanguineus s. s.* (af). These facts suggest the presence of an association between the morphologic cluster *R. sanguineus* Intermediate and *R. sanguineus* and quantitative cluster 1.

These associations come out as a natural result by looking at the data presented on table 6 and at the morphological characteristics that constitute the description of this quantitative cluster. So, quantitative cluster 1 is associated with elements that present large scutums, large capitulum, with average size porose areas, large spiracles with high tails, high and wide sclerites and wide genital pore aperture. These characteristics can be found in the morphologic clusters *R. sanguineus s. s., R. sanguineus* Intermediate and *R. sanguineus* T2, as evidenced by the data presented by Table 9 and descriptions of these morphological clusters, that resulted from that.

As the morphologic clusters *R. sanguineus s. s.*, *R. sanguineus* Intermediate and *R. sanguineus* T2 are quite similar from a morphological point of view, sharing plenty of morphological features, it seems logical their association in the same quantitative cluster.

Also in the quantitative clusters 1, it is possible to evidence the presence of some elements that were classified as *R. sanguineus* T1 and *R. pusilus*, despite the fact that it is almost negligible once it only represents 2% and 4% respectively. Their presence comes as an odd result, especially in the case of *R. pusilus* that don't have much in common with the others morphologic groups included in the quantitative cluster 1. The most likely explanation is that these six specimens of *R. pusillus* were inserted in this cluster as an outlier, as a consequence of the fact that this quantitative cluster is significantly large, once it contains 147 females, 64% of the total feminine sample considered in this study.

The information provided in table 8 shows that within the 52 specimens belonging to the quantitative cluster 2, seven (13,5%) were classified as *R. sanguineus s. s.*, seven (13,5%) as *R. sanguineus* Type 1, eight (15,4%) as *R. sanguineus* Type 2, four (7,7%) as *R. sanguineus* Intermediate, 14 (26,9%) as *R. pusillus* and 12 (23,1%) were classified as *R. turanicus* (fig. 42).

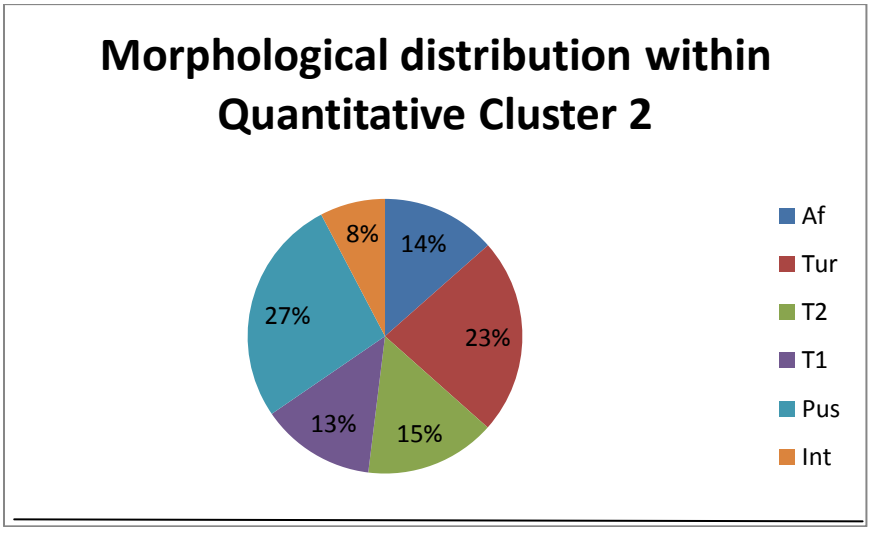


Fig. 42: Morphologic distribution within the Quantitative Cluster 2- Graphic that relates the taxonomic clusters obtained by morphologic analysis, with the qualitative cluster 2 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 7 females (13,5%); **T1:** *R. sanguineus* Type 1 – 7 females (13,5%); **T2:** *R sanguineus* type 2 – 8 females (15,4%); **Int:** *R. sanguineus* Int – 4 females (7,7%); **Tur:** *R. turanicus* – 12 females (23,1%); **Pus:** *R. pusillus* – 14 females (26,9%).

Data on Fig. 42, shows the presence of six different morphological groups within the quantitative cluster 2. The existence of so many morphological groups, such as *R. sanguineus* and *R. turanicus* suggests that this is a quantitative cluster, with large intra-specific variation, and therefore it is difficult to describe its main morphological characteristics that define it.

This quantitative cluster does not evidence an obvious association, like the one described between quantitative cluster 1 and *R. sanguineus* T2. However the fact that *R. turanicus* and *R. pusillus* are the morphologic groups with a more meaningful representation, suggests an association between this morphologic cluster and quantitative cluster 2.

The 12 specimens classified as *R. turanicus* constitutes 100% of the total sample of the *R. turanicus* morphologic cluster, meaning their entire inclusion within the quantitative cluster 2, and confirming the existence of an association between this morphologic cluster and the quantitative cluster 2. A similar situation occurs with 14 individuals classified as *R. pusillus* representing 70% of the total sample of the morphologic cluster *R. pusillus*, which suggest the presence of an association between the morphologic cluster *R. pusillus* and quantitative cluster 2.

The association between the quantitative Cluster 2 and the morphologic cluster, *R. pusillus*, comes out as a logical result because quantitative cluster 2 is associated with the specimens that show the smallest scutum and capitulum of the sample; the same situation occurs with *R. pusilus* morphological cluster. Besides these similarities they both have spiracles with low and wide tails and narrow genital pore aperture.

The association between the quantitative Cluster 2 and the morphologic cluster *R. turanicus* is supported by the fact that this quantitative cluster is associated with spiracles with small angles, low and wide tails, and the narrowest genital pore aperture of the sample; these morphological features can be found in the morphologic cluster *R. turanicus*, as evidenced on Table 9 and descriptions of these morphological clusters, that resulted from that.

Simultaneously the fact that the morphologic clusters *R. pusillus* and *R. turanicus* are associated with the same morphologic cluster is a natural result, despite the differences on some morphologic measurements (*R. pusillus* is significantly smaller than *R. turanicus*) they

still share several morphological features, namely spiracles with small angles, low and wide tails and narrow genital pore aperture.

Data on fig 42 also enlightens why quantitative cluster 2 is the one with the smallest scutums and the smallest capitulum, since the elements that belong to the *R. pusillus* morphologic cluster were included in this quantitative cluster, as this species is characterized by smaller dimensions of the various structures that composes its bodies. The inclusion of these individuals in this cluster led to a significant diminuishing of the dimensions average of certain morphological characteristics.

In turn, data show that within the 34 specimens belonging to the quantitative cluster 3, 18 (48,6%) were classified as *R. sanguineus* type 1, 15 (40,5%) as *R. sanguineus* Type 2 and four (10,9%) as *R. sanguineus s. s.* (fig. 43).

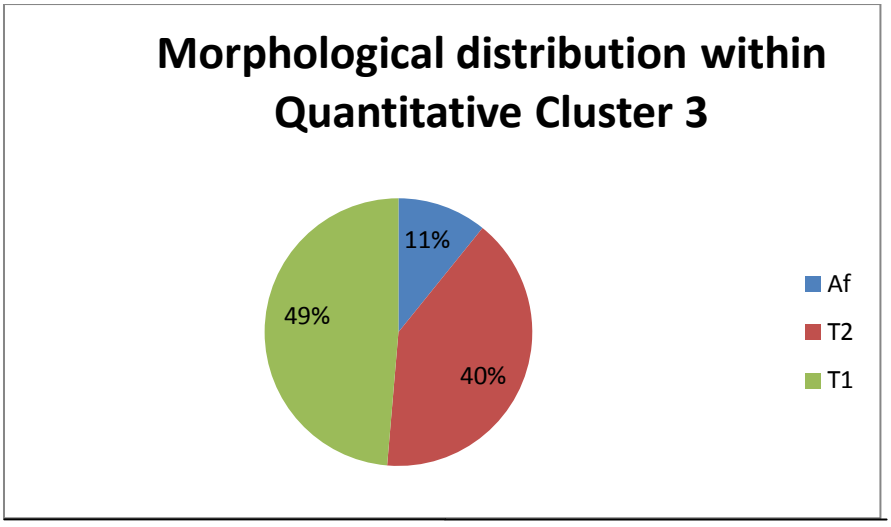


Fig. 43: Morphologic distribution within the Quantitative Cluster 3- Graphic that relates the taxonomic clusters obtained by morphologic analysis, with the quantitative cluster 3 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* – 4 females (10,9%); **T1:** *R. sanguineus* Type 1 – 18 females (48,6%); **T2:** *R sanguineus* type 2 – 15 females (40,5%).

Data on Fig. 43 suggest that there are only three morphological groups in this cluster from which *R. sanguineus* T1 and *R. sanguineus* T2 dominate, so it is possible to conclude that there is an association between both these morphologic clusters and quantitative cluster 1.

The association between *R. sanguineus* T1 morphologic cluster and quantitative cluster 3 result once that this quantitative cluster is related to elements that exhibit, medium size scutum, average sized porose areas, spiracles with large angles and with high and narrow

tails, a wide genital aperture and low and narrow sclerites; morphological features, that can be found in the morphologic cluster *R. sanguineus* T1, as evidenced by the data on Table 9 and descriptions of these morphological clusters, that can be infered from that.

The association between *R. sanguineus* T2 morphologic cluster and quantitative cluster 3, also comes as a natural result once this quantitative cluster is associated with elements that are characterized by average sized porose areas, large spiracles with high tails, large angles and wide genital pore aberture; morphological features, that can be found in the morphologic cluster *R. sanguineus* T2. The fact that the morphologic clusters *R. sanguineus* T2 and *R. sanguineus* T1 are quite similar from a morphological point of view, once they share some morphological features, which justify their inclusion in the same quantitative cluster. However this association between *R. sanguineus* T2 and quantitative cluster 3 is weaker than the association established between *R. sanguineus* T2 and quantitative cluster 3.

This quantitative cluster also presents in its constitution some specimens that were classified as *R. sanguineus s. s.* which is explained, by morphological features shared between *R. sanguineus* T1 and *R. sanguineus* T2.

Data show that within the 49 specimens belonging to the qualitative cluster 1, 19 (38,7%) were classified as *R. sanguineus sensu stricto* (Af), 12 (24,5%) as *R. sanguineus* Type 1 and 18 (36,7%) as *R. pusillus* (fig.44).

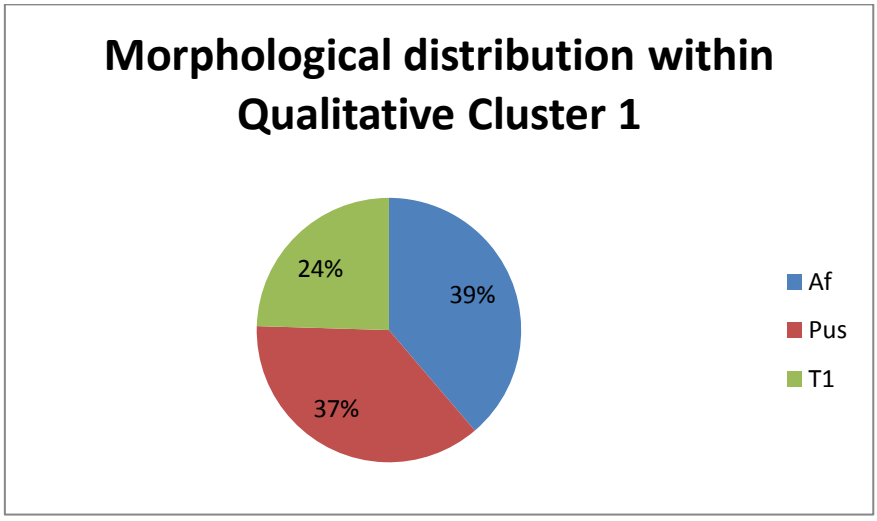


Fig. 44: Morphologic distribution within the Qualitative Cluster 1- Graphic that relates the morphologic clusters obtained by morphologic analysis with the qualitative cluster 1 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* –19 females (38,7%); **T1:** *R. sanguineus* Type 1 – 12 females (24,5%); **Pus:** *R. pusillus* – 18 females (36,7%).

Data on Fig. 44 shows only three morphological groups in this cluster and it seems that there is an association between each one of this these three morphologic clusters and qualitative cluster 1.

However some of these associations are stronger than others. The fact that 18 elements within this cluster were classified as *R. pusillus* means that 90% was included in this qualitative cluster and the fact that that such large portion of this morphologic cluster was included in qualitative cluster 1, suggest that this is strongest association within this qualitative cluster.

This does not occur either with *R. sanguineus s. s.* or with *R. sanguineus* T1, once the specimens included within this qualitative cluster only represent 49% and 43% of the samples that compose their respective morphologic clusters. Despite that fact this quantitative cluster is still the one that presents by far most specimens of *R. sanguineus* and *R. sanguineus* T1 and also the one where both morphologic clusters are more represented, suggesting an association among *R. sanguineus s. s.* and *R. sanguineus* T1 and this qualitative cluster. However these associations are weaker than the one that occurs between *R. pusillus* and quantitative cluster 1 it is also easy to conclude that the association between *R. sanguineus* 1 and quantitative cluster 1 is the weakest of them all.

R. sanguineus s. s. and *R. sanguineus* T1 are associated with the same quantitative cluster, once the main quantitative variable is "Genital aperture shape" and both these morphologic clusters exhibit the pattern 2. In addiction *R. pusillus* is associated with the same qualitative cluster which is also explainable. In fact, regarding quantitative variables, this morphologic group is closer to *R. turanicus,* than *R. sanguineus*, however, when it comes to qualitative variables, the most important one is "Genital aperture shape" and *R. pusilus* shows the pattern 1 that is very similar to the pattern 2.

The information provided in table 8 shows that within the 152 specimens belonging to the qualitative cluster 2, 13 (8,6%) were classified as *R. sanguineus s. s.*, nine (5,9%) as *R. sanguineus* Type 1, 104 (68,4%) as *R. sanguineus* Type 2, 17 (11,2%) as *R. sanguineus* Intermediate and nine (5,9%) as *R. turanicus* (fig 43).

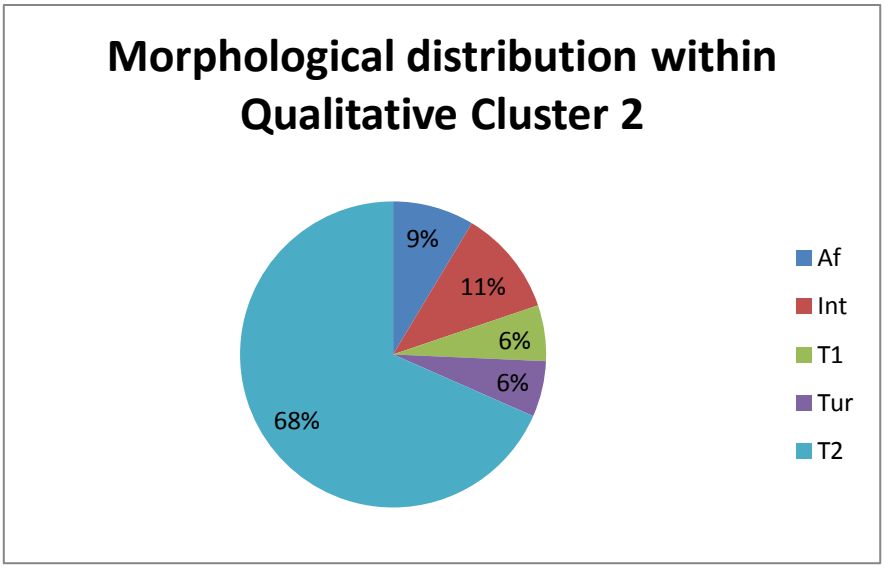


Fig. 45: Morphologic distribution within the Qualitative Cluster 2- Graphic that relates the morphologic clusters obtained by morphologic analysis with the qualitative cluster 1 obtained by statistical analysis**: Af:** *R. sanguineus sensu stricto* –13 females (8,6%); **T1:** *R. sanguineus* Type 1 – 9 females (5,9%); **T2:** *R sanguineus* type 2 – 104 females (68,4%); **Int:** *R. sanguineus* Int – 17 females (11,2%); **Tur:** *R. turanicus* – 9 females (5,9%).

Data presented on Fig. 45 show the presence of five different morphological groups within the quantitative cluster 2. The presence of so many morphological groups, as *R. sanguineus* and *R. turanicus* suggests a quantitative cluster with large intra-specific variation making difficult to describe the morphological characteristics that define it.

Nevertheless there is one group, *R. sanguineus* T2 that clearly dominate over the others, which indicates an association between this morphological clusters and qualitative cluster 2. The presence of many morphologic groups within this qualitative Cluster, it is associated with *R. sanguineus* T2 that is one of the intermediate forms, which shares morphological characteristics with *R. sanguineus* and *R. turanicus*. This allows the formation of a cluster characterized by a wide range of characteristics, which enables the fitting of such distinctive morphological groups. It is a very large cluster once it contains 152 females, 64,4% of the total feminine sample considered in this study, suggesting that some of the specimens from the others morphologic clusters where inserted in this quantitative cluster as outliers.

However, despite the association with *R. sanguineus* T2 being the strongest presented by this cluster, when analyzing the constitution of this quantitative clusters more closely, it is possible to note the presence of 17 specimens that were classified as *R. sanguineus* Intermediate, that is 81% of the total sample of the *R. sanguineus* Intermediate morphologic cluster. Nine individuals were classified as *R. turanicus* that represents 75% of the total

sample of the morphologic cluster *R. turanicus.* As large percentages of these morphologic clusters were included in this qualitative cluster suggest an association among the morphologic cluster *R. sanguineus* Intermediate and *R. turanicus* and qualitative cluster 2.

The information regarding the qualitative cluster 3 shows that within the 35 specimens included in the qualitative cluster 3, seven (20,0%) were classified as *R. sanguineus s. s.*, seven (20,0%) as *R. sanguineus* Type 1, 12 (34,3%) as *R. sanguineus* Type 2, four (11,4%) as *R. sanguineus* Intermediate, three (8,6%) as *R. turanicus*, 2 (5,9%) and two (5,7 %) as *R. pusillus* (fig.46).

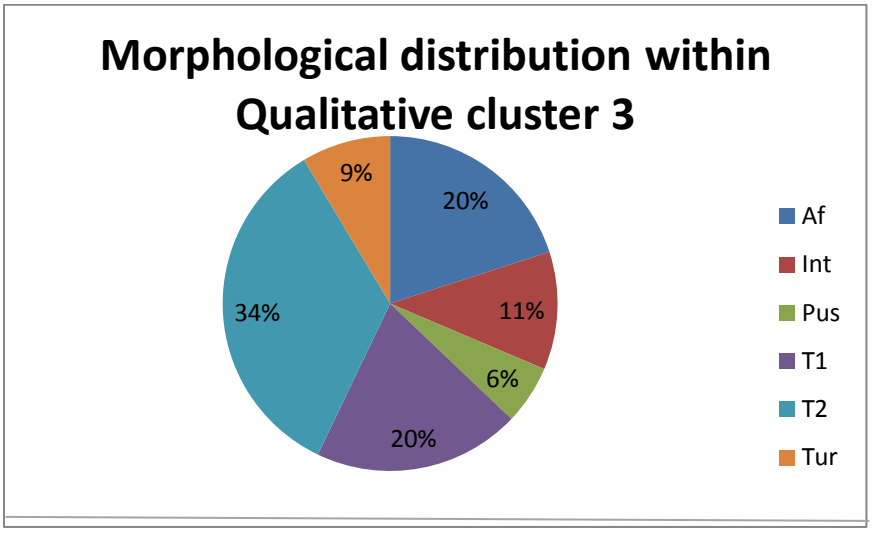


Fig. 46: Morphologic distribution within the Qualitative Cluster 3- Graphic that relates the morphologic clusters obtained by morphologic analysis with the qualitative cluster 3 obtained by statistical analysis**: Af:** *R. sanguineus sensu strito* – 7 females (20,0%); **T1:** *R. sanguineus* Type 1 – 7 females (20,0%); **T2:** *R sanguineus* type 2 – 12 females (34,4%); **Int:** *R. sanguineus* Int – 4 females (11,4%); **Tur:** *R. turanicus* – 3 females (8,6%); **Pus:** *R. pusillus* – 2 females (5,7%).

Data presented on Fig. 46 show the presence of six different morphological groups within the quantitative cluster 2. The presence of so many morphological groups, as *R. sanguineus s. s.* and *R. turanicus* suggests that this is a quantitative cluster with large intra-specific variation, and therefore it is difficult to define the morphological characteristics that descrive it.

However, it appears to exist an association between the *R. sanguineus* T2 morphological cluster and qualitative cluster 3, which may explain the presence of many morphologic groups within this qualitative cluster, since it is associated with *R. sanguineus* T2, one of the intermediate forms that shares morphological features with *R. sanguineus* and *R. turanicus*. This fact allows the formation of a cluster characterized by a wide range of characteristics, which enables the fitting of such distinctive morphological groups.

However, this association is weaker that the one described between *R. sanguineus* T2 and the qualitative cluster 2, once 104 (89%) of the specimens that compose this morphologic cluster were included in qualitative cluster 2, what indicates a very strong association.

Qualitative cluster 3 does not present a strong association like the one that was described between *R. sanguineus* T2 and the qualitative cluster 2, or the one described between *R. pusillus* and the qualitative cluster 1, in conjunction with the presence of a large quantity of specimens from many morphological groups, as distinct from each other such as *R. sanguineus s. s.* and *R. turanicus*, suggesting that the qualitative variables did not differentiate the specimens so well, as in the other two qualitative clusters. Though this qualitative cluster presenting less differentiation capacity and less characterization power.

4.3 Genetic Analysis

After a detailed morphological and statistical study, 146 ticks representing the various quantitative, qualitative and morphological clusters formed in the previous analyzes perfomerd were chosen, in order to characterize the genetic variability in this sample by using molecular markers 12S and 16S. From those ticks, a total of 73 sequences, in optimal conditions, were acquired 34 from marker 16S and 39 from the marker 12S, respectively.

Table 16, (showed in appendices) displays the absolute nucleotide differences and the p-distance between every sequence obtained with the 12S molecular marker, revealing the presence of 5 different haplotypes that are evidenced in fig. 47.

In turn, table 17 (presented in appendices) displays the absolute nucleotide differences and the p-distance among all sequences obtained with the 16S molecular marker, revealing the presence of 10 different haplotypes that are mentioned fig. 48.

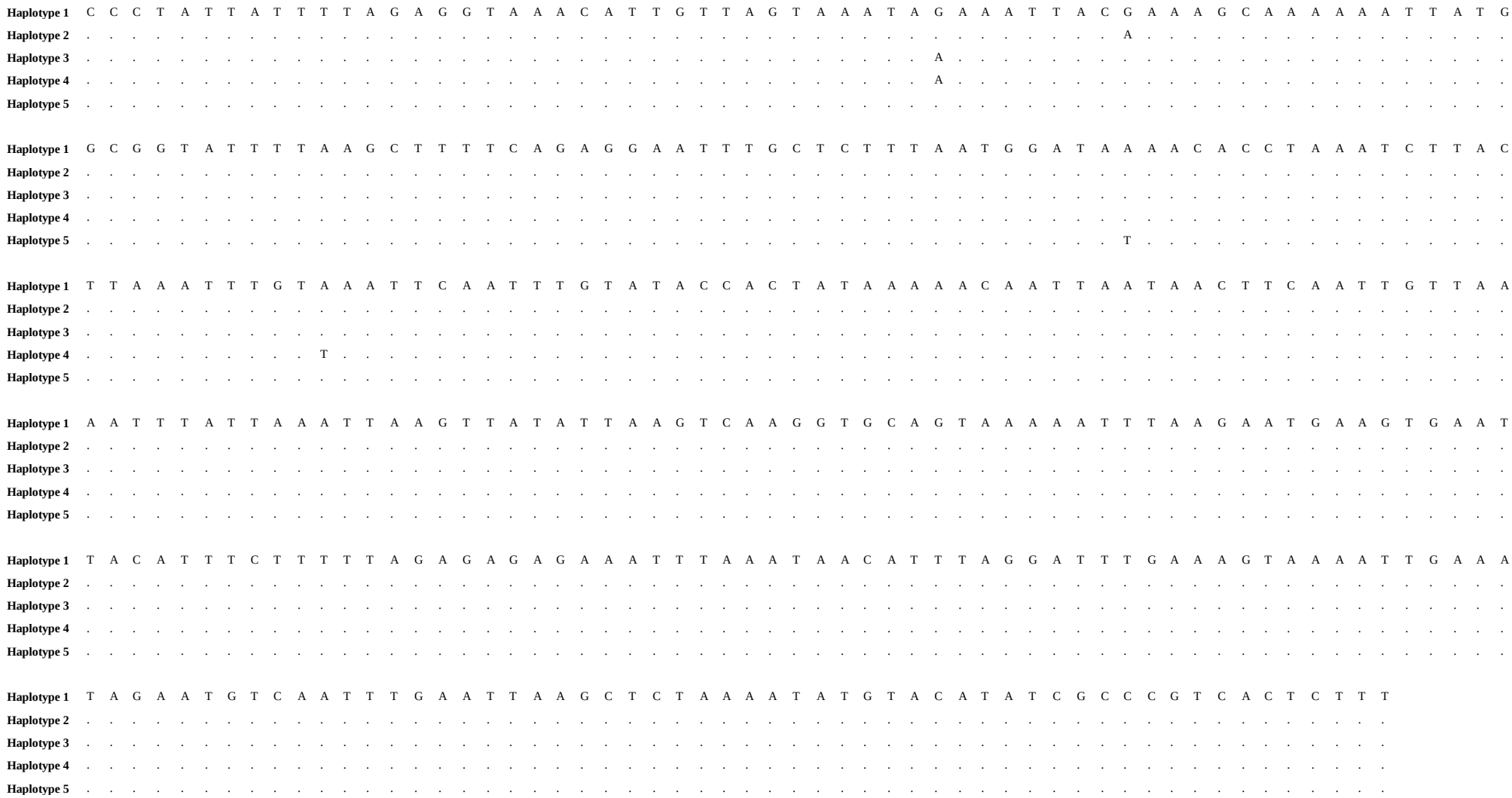


Fig. 47: Morphologic Alignment of nucleotide sequences (5`-3`) of the 12S rDNA gene of the ten haplotypes found in the specimens considered in this study. A point indicates that the sequence at that point is identical to the sequence of the top. A hyphen indicates a gap.

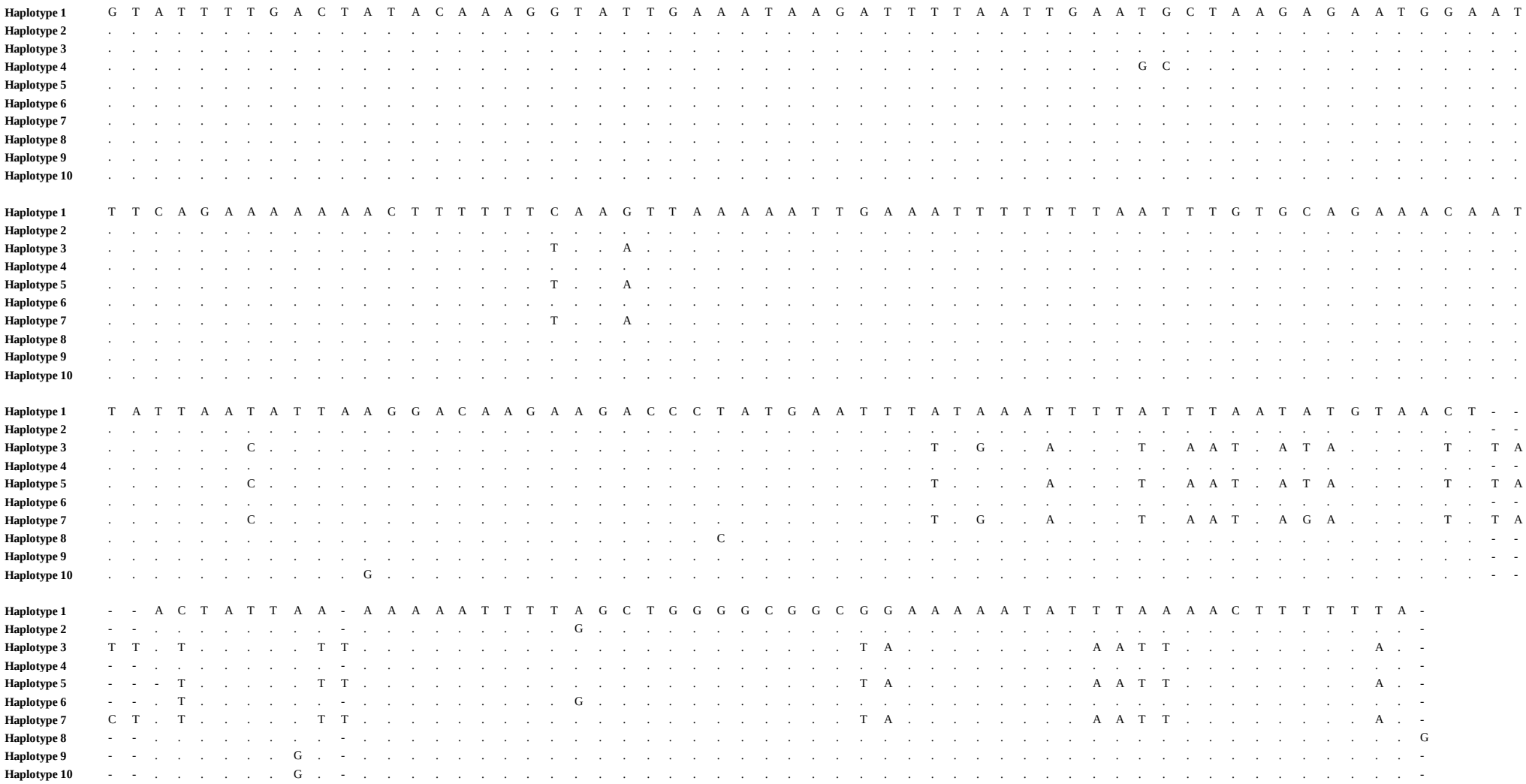


Fig. 48: Morphologic Alignment of nucleotide sequences (5`-3`) of the 16S rDNA gene of the ten haplotypes found in the specimens considered in this study. A point indicates that the sequence at that point is identical to the sequence of the top. A hyphen indicates a gap.

Data on the characterization of the variability by several haplotypes obtained with the 12S and the 16S markers are showed in tables 10 and 11, respectively.

Table 10 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in italics, between the five haplotypes presented by the 12S rRNA gene in this study.

Haplotypes 12S					
	Hap 1	**Hap 2**	**Hap 3**	**Hap 4**	**Hap 5**
Hap 1		*0,28%*	*0,28%*	*0,55%*	*0,28%*
Hap 2	**1**		*0,55%*	*0,83%*	*0,55%*
Hap 3	**1**	**2**		*0,28%*	*0,55%*
Hap 4	**2**	**3**	**1**		*0,83%*
Hap 5	**1**	**2**	**2**	**3**	

Note: P-distance= (number of different nucleotides/ Total number of analyzed nucleotides. Hap- Haplotype.

The mean p-distance between the haplotypes obtained ranged from 0,28% to 0,83%, and the absolute number of absolute differences, varried from 1 to 3, numbers that indicate, some intra-specific variation however, these are low values.

Table 11 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in (italics), between the ten haplotypes presented by the 16S rDNA gene in this study.

Haplotypes 16S										
	Hap 1	**Hap 2**	**Hap 3**	**Hap 4**	**Hap 5**	**Hap 6**	**Hap 7**	**Hap 8**	**Hap 9**	**Hap 10**
Hap 1		*0,43%*	*9,83*	*0,85%*	*9,44%*	*0,85%*	*9,83%*	*0,43%*	*0,43%*	*0,85%*
Hap 2	**1**		*10,26%*	*1,28%*	*9,87%*	*0,43%*	*10,26%*	*0,85%*	*0,85%*	*1,28%*
Hap 3	**23**	**24**		*10,68%*	*0,42%*	*9,83%*	*0,84%*	*10,26%*	*10,26%*	*10,68%*
Hap 4	**2**	**3**	**12**		*10,30%*	*1,71%*	*10,68%*	*1,28%*	*1,28%*	*1,71%*
Hap 5	**22**	**23**	**1**	**24**		*9,44%*	*0,85%*	*9,87%*	*9,87%*	*10,30%*
Hap 6	**2**	**1**	**23**	**4**	**22**		*9,83%*	*1,28%*	*1,28%*	*1,71%*
Hap 7	**23**	**24**	**2**	**25**	**2**	**23**		*10,26%*	*10,26%*	*10,68%*
Hap 8	**1**	**2**	**24**	**3**	**23**	**3**	**24**		*0,85%*	*1,28%*
Hap 9	**1**	**2**	**24**	**3**	**23**	**3**	**24**	**2**		*0,43%*
Hap 10	**2**	**3**	**25**	**4**	**24**	**4**	**25**	**3**	**1**	

Note: P-distance= (number of different nucleotides/ Total number of analyzed nucleotides. Hap- Haplotype.

The mean p-distance between the haplotypes obtained ranged from 0,43% to 10,68%, and the absolute number of absolute differences varried from 1 to 24, however the highest values obtained correspond to haplotypes 3, 5 and 7, and these three haplotypes were isolated in specimens that belonged to *R. pusillus* species, that were included as an control group, so these numbers don't really translate the true numbers of variability in our sample. However by excluding this results, it is possible to observe that the mean p-distance obtained varied from 0,43% to 1,71%, and the number of absolute differences, ranged from 1 to 4.

These values are more interesting than the ones obtained between the haplotypes with the marker 12S, these values indicate some intra-specific variation, although it is not enough to consider this variability, to justify a classification as different species. For that values between 5% and 8% should be acquired [18, 86, 87]. Nevertheless this numbers, correspond to a very interesting intra-specific variation.

The next step was to understand how the sequences obtained in this study would relate with others sequences, from other studies and others countries, which is showed in tables 12 and 13.

By analyzing table 12 it is possible to notice that the p-distances between 5 haplotypes obtained in this study, with the molecular marker 12S and the various sequences taken from the GenBank isolated in other geographical locations, it is possible to infer several elations. When comparing the five haplotypes acquired in this work with sequences from other studies previously conducted in Portugal (KC243805), (KC243806) and (KC243807) it is possible to observe that the p-distances are between 0,00% and 1,22%, values indicating the existence of intra-specific variability. More than that, it indicates the absence of enough genetic differences that justify the classification as a different species. This can be considered as one expected result, attending that this sequences the same geographic origin. When analyzing the haplotypes obtained in these study with the sequences isolated in Spain (KC243802) it is observable a slight increase in p-distances, which lead to values that ranged between 0,61% and 1,51%, indicating an increase in intra-specific variability, again this is not enough to justify the classification as a distinct species. This can also be considered a normal result, because in spite of being sequences with origins in different countries, Portugal and Spain are countries that share the same geo-environmental conditions. A similar situation, occurs when comparing the 5 haploypes from this study with sequences obtained in France (JX304744) in

that case the p-distance range from 0,00% to 0,61% points to that these specimens are very similar to the ones found in France, probably for similar reasons, already mentioned above when comparing with the Spanish sequences. The comparison with sequences from Greece (KC243796), (KC243799), (KC243793), (KC243801), (KC243822) and (KC243817) allow getting p-distance values in the order of 7,01% and 7,93%, as well as a great variability, which could already reflect an inter-specific relationship. This is also an expectable result because the Greek sequences were obtained in *R. sanguineus*, belonging to morfotype 1, and Spain, Portugal and France are often associated with ticks that belong to the morfotype 2, which explains the lower p-distance values, when comparing the haplotypes from this study with sequences from these countries, and higher when compared with sequences isolated in specimens coming from Greece. An inter-specific relation is also observable between the 5 haplotypes in this study and the sequences isolated in *R. turanicus* from Italy (KC243817), (KC243822), (KC243821), Israel, (AF15001), and Switzerland, (AF483243), presenting p-distance values that range from 6,40% to 8,53%. It is also noteworthy, that the haplotype with the lowest p-distance to the *R. turanicus* group was haplotype 3 that was obtained from a specimen previously classified as a *R. turanicus* in the morphologic study. Finally it is also noticible that the highest values of p-distance ranged from 9,15% to 9,76% which were establish between the haplotypes acquired in this study, and the sequences from *R. sanguineu*s isolated in Brazil (AY559842), Argentina, (JX206968), (JX206971) Mozambique, (JX206978) and South Africa, (JX206998). These specimens of *R. sanguineus* belong to the tropical linage, also known as the northern linage. This is also a normal result because, although these specimens from tropical countries and those found in the Europeans countries are classified as *R. sanguineus*, they are associated with very different morphotypes, which is translated in high p-distances. [32, 86–88]

By analyzing the data on table 13 in particular the p-distances between the 10 haplotypes obtained in this study with the molecular marker 16S and the various sequences taken from the GenBank, isolated in other geographical locations, it is possible to achieve several conclusions. Particularly when comparing those haplotypes acquired with sequences from others investigations previously conducted in Portugal (KC243844), (KC243845) and (KC243846), by excluding the haplotypes 3, 5 and 7 that were obtained from individuals that belong to the *R. pusillus* (control group), it is possible to observe that the p-distances are between 0,43% and 2,55%, suggesting the presence of an interesting intra-specific variability, although they are not high enough to justify the classification as different species. When

analyzing the haplotypes obtained in this study with the sequences isolated in Spain, (KC243843), (GU553081), p-distance values range between 0,43% and 2,13%, indicating a similar level of intra-specific variability. A similar situation occurs when comparing the ten haploypes from this study with sequences obtained in France, in that case the p-distance range from 0,43% to 2,55% . The compatison with sequences from Greece, (KC243841), (KC243840), (KC243842) and (KC243839) shows p-distance values on the order of 4,68% and 7,66%, which already reflects great variability and an inter-specific relationship. Another inter-specific relation is also observable between the haplotypes acquired in this study and the sequences isolated in *R. sanguineus* from tropical countries such as Brazil, (JX206980) Mozambique (JX195173) and South Africa (GU553079), which evidences p-distance values varying from 7,23% to 9,79%. The p-distance values obtained by comparing the haplotypes from this study with the sequences isolated in Italians *R. turanicus* (KC243856), (KC243858) and (KC243860) range between 8,51% and 10,51% also suggest high variability and points to an inter-specific relation. Finally, it is also noticible that the highest values of p-distance ranged among 10,21% and 11,06% and were establish between, the 3 haplotypes isolated in ticks that belonged to the *R. pusillus*. Despite some differences these results are very similar to the ones displayed by table 12, therefore are explicable precisely for the reasons detailed above.

The fact that the haplotypes acquired in this study, when compared to sequences previously isolated in France, Spain and Portugal point to lower p-distance values; when compared to sequences isolated in Greece, Italy and tropical countries suggest that the haplotypes found in this study are genetically closer to the morphotype 2 and consequently more distant to the morphotype 1, morphotype *R. turanicus* and morphotype *R. sanguineus sensu lato*, associated with Greece, Italy and tropical countries respectively [32].

Table 12 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in italics, between the haplotypes obtained in this study, and several R. s. and R. tur isolated from different origins presented by the 12S rDNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. Hap 1		*0,30%*	*0,61%*	*0,30%*	*0,30%*	*0,30%*	*9,45%*	*9,45%*	*9,45%*	*9,45%*	*9,45%*	*7,62%*	*7,62%*	*7,62%*	*7,62%*	7,62%	*0,00%*	*0,61%*	*0,91%*	*1,22%*	*8,23%*	*7,62%*	*7,62%*	*7,93%*	*7,01%*	*9,45%*
2 .Hap 2	**1**		*0,30%*	*0,61%*	*0,61%*	*0,00%*	*9,15%*	*9,15%*	*9,15%*	*9,15%*	*9,15%*	*7,32%*	*7,32%*	*7,32%*	*7,32%*	7,32%	*0,30%*	*0,30%*	*0,61%*	*0,91%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*6,71%*	*9,15%*
3. Hap 3	**1**	**2**		*0,91%*	*0,91%*	*0,30%*	*9,15%*	*9,15%*	*9,15%*	*9,15%*	*9,15%*	*7,01%*	*7,01%*	*7,01%*	*7,01%*	7,01%	*0,61%*	*0,61%*	*0,30%*	*0,61%*	*7,62%*	*7,01%*	*7,01%*	*7,32%*	*6,40%*	*9,15%*
4. Hap 4	**2**	**3**	**1**		*0,61%*	*0,61%*	*9,76%*	*9,76%*	*9,76%*	*9,76%*	*9,76%*	*7,93%*	*7,93%*	*7,93%*	*7,93%*	7,93%	*0,30%*	*0,91%*	*1,22%*	*1,52%*	*8,54%*	*7,93%*	*7,93%*	*8,23%*	*7,32%*	*9,76%*
5. Hap 5	**1**	**2**	**2**	**3**		*0,61%*	*9,76%*	*9,76%*	*9,76%*	*9,76%*	*9,76%*	*7,93%*	*7,93%*	*7,93%*	*7,93%*	7,93%	*0,30%*	*0,91%*	*1,22%*	*1,52%*	*8,54%*	*7,93%*	*7,93%*	*8,23%*	*7,32%*	*9,76%*
6. R. s. France (JX304744)	**1**	**2**	**0**	**1**	**2**		*9,15%*	*9,15%*	*9,15%*	*9,15%*	*9,15%*	*7,32%*	*7,32%*	*7,32%*	*7,32%*	7,32%	*0,30%*	*0,30%*	*0,61%*	*0,91%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*6,71%*	*9,15%*
7. R. s. Mozambique (JX206978)	**31**	**32**	**30**	**30**	**32**	**30**		*0,91%*	*0,91%*	*0,91%*	*0,30%*	*7,32%*	*6,40%*	*7,3%2*	*7,3%2*	6,71%	*9,45%*	*9,45%*	*9,45%*	*9,15%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*7,01%*	*2,74%*
8 .R. s. af S. Africa (JX206998)	**31**	**32**	**30**	**30**	**32**	**30**	**3**		*0,00%*	*0,00%*	*0,61%*	*7,01%*	*6,10%*	*7,01%*	*7,01%*	6,40%	*9,45%*	*9,45%*	*9,45%*	*9,15%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*7,01%*	*2,44%*
9. R. s. Brazil (AY559842)	**31**	**32**	**30**	**30**	**32**	**30**	**3**	**0**		*0,00%*	*0,61%*	*7,01%*	*6,10%*	*7,01%*	*7,01%*	6,40%	*9,45%*	*9,45%*	*9,45%*	*9,15%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*7,01%*	*2,44%*
10. R. s. Argentina (JX206968)	**31**	**32**	**30**	**30**	**32**	**30**	**3**	**0**	**0**		*0,61%*	*7,01%*	*6,10%*	*7,01%*	*7,01%*	6,40%	*9,45%*	*9,45%*	*9,45%*	*9,15%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*7,01%*	*2,44%*
11. R. s. Argentina (JX206971)	**31**	**32**	**30**	**30**	**32**	**30**	**1**	**2**	**2**	**2**		*7,01%*	*6,10%*	*7,01%*	*7,01%*	6,40%	*9,45%*	*9,45%*	*9,45%*	*9,15%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*7,01%*	*2,44%*
12. R. s. T1 Grecia (KC243796)	**25**	**26**	**24**	**23**	**26**	**24**	**24**	**23**	**23**	**23**	**23**		*2,44%*	*0,00%*	*0,00%*	1,22%	*7,62%*	*7,62%*	*7,32%*	*7,01%*	*7,32%*	*6,71%*	*6,71%*	*7,01%*	*6,71%*	*5,79%*
13. R. s. T1 Grecia (KC243801)	**25**	**26**	**24**	**23**	**26**	**24**	**21**	**20**	**20**	**20**	**20**	**8**		*2,44%*	*2,44%*	1,83%	*7,62%*	*7,62%*	*7,32%*	*7,01%*	*6,71%*	*6,10%*	*6,10%*	*6,40%*	*6,10%*	*4,88%*
14. R. s. T1 Grecia (KC243897)	**25**	**26**	**24**	**23**	**26**	**24**	**24**	**23**	**23**	**23**	**23**	**0**	**8**		*0,00%*	1,22%	*7,62%*	*7,62%*	*7,32%*	*7,01%*	*7,32%*	*6,71%*	*6,71%*	*7,01%*	*6,71%*	*5,79%*
15. R. s. T1 Grecia (KC243799)	**25**	**26**	**24**	**23**	**26**	**24**	**24**	**23**	**23**	**23**	**23**	**0**	**8**	**0**		1,22%	*7,62%*	*7,62%*	*7,32%*	*7,01%*	*7,32%*	*6,71%*	*6,71%*	*7,01%*	*6,71%*	*5,79%*
16. R. s. T1 Grecia (KC243793)	**25**	**26**	**24**	**23**	**26**	**24**	**22**	**21**	**21**	**21**	**21**	**4**	**6**	**4**	**4**		*7,62%*	*7,62%*	*7,32%*	*7,01%*	*7,01%*	*6,40%*	*6,40%*	*6,71%*	*6,40%*	*5,18%*
17. R. s. T2 Portugal (KC243807)	**0**	**1**	**1**	**2**	**1**	**1**	**31**	**31**	**31**	**31**	**31**	**25**	**25**	**25**	**25**	**25**		*0,61%*	*0,91%*	*1,22%*	*8,23%*	*7,62%*	*7,62%*	*7,93%*	*7,01%*	*9,45%*
18. R. s. T2 Portugal (KC243806)	**2**	**3**	**1**	**2**	**3**	**1**	**31**	**31**	**31**	**31**	**31**	**25**	**25**	**25**	**25**	**25**	**2**		*0,30%*	*0,61%*	*8,23%*	*7,62%*	*7,62%*	*7,93%*	*7,01%*	*9,45%*
19. R. s. T2 Portugal (KC243807)	**3**	**4**	**2**	**1**	**4**	**2**	**31**	**31**	**31**	**31**	**31**	**24**	**24**	**24**	**24**	**24**	**3**	**1**		*0,30%*	*7,93%*	*7,32%*	*7,32%*	*7,62%*	*6,71%*	*9,45%*
20. R. s. T2 Spain (KC243802)	**4**	**5**	**3**	**2**	**5**	**3**	**30**	**30**	**30**	**30**	**30**	**23**	**23**	**23**	**23**	**23**	**4**	**2**	**1**		*7,62%*	*7,01%*	*7,01%*	*7,32%*	*6,40%*	*9,15%*
21. R. tur Italia (KC243821)	**27**	**28**	**26**	**25**	**28**	**26**	**26**	**26**	**26**	**26**	**26**	**24**	**22**	**24**	**24**	**23**	**27**	**27**	**26**	**25**		*0,61%*	*0,61%*	*0,30%*	*2,13%*	*6,71%*
22. R. tur Italia (KC243817)	**25**	**26**	**24**	**23**	**26**	**24**	**24**	**24**	**24**	**24**	**24**	**22**	**20**	**22**	**22**	**21**	**25**	**25**	**24**	**23**	**2**		*0,61%*	*0,30%*	*1,52%*	*6,10%*
23. R. tur Italia (KC243822)	**25**	**26**	**24**	**23**	**26**	**24**	**24**	**24**	**24**	**24**	**24**	**22**	**20**	**22**	**22**	**21**	**25**	**25**	**24**	**23**	**2**	**2**		*0,30%*	*2,13%*	*6,10%*
24. R. tur Israel (AF15001)	**26**	**27**	**25**	**24**	**27**	**25**	**25**	**25**	**25**	**25**	**25**	**23**	**21**	**23**	**23**	**22**	**26**	**26**	**25**	**24**	**1**	**1**	**1**		*1,83%*	*6,40%*
25. R. tur Switerzeland (AF48317)	**23**	**24**	**22**	**21**	**24**	**22**	**23**	**23**	**23**	**23**	**23**	**22**	**20**	**22**	**22**	**21**	**23**	**23**	**22**	**21**	**7**	**5**	**7**	**6**		*6,40%*
26. R. tur Zimbabwe (AF50017)	**31**	**32**	**30**	**30**	**32**	**30**	**9**	**8**	**8**	**8**	**8**	**19**	**16**	**19**	**19**	**17**	**31**	**31**	**31**	**30**	**22**	**20**	**20**	**21**	**21**	

Note: Hap- Haplotype; (R. s.) *R. sanguineus*; (R.tur) *R. turanicus.*

Table 13 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in (italics), between the haplotypes obtained in this study, and several R. s. and R. tur isolated from different origins presented by the 16S rDNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. Hap 1		*1,70%*	*10,21%*	*0,85%*	*10,21%*	*0,85%*	*10,21%*	*0,43%*	*1,28%*	*1,70%*	*9,36%*	*9,36%*	*8,94%*	*9,36%*	*9,79%*	*9,79%*	*6,38%*	*6,81%*	*6,38%*	*6,81%*	*1,28%*	*1,70%*	*0,85%*	*1,70%*	*1,28%*	*1,28%*	*1,28%*
2. Hap 2	**4**		*11,06%*	*2,55%*	*11,06%*	*1,70%*	*11,06%*	*2,13%*	*0,43%*	*0,85%*	*7,66%*	*7,66%*	*7,23%*	*8,94%*	*8,94%*	*8,94%*	*4,68%*	*5,11%*	*4,68%*	*5,11%*	*0,43%*	*0,00%*	*0,85%*	*0,85%*	*0,43%*	*0,43%*	*0,43%*
3. Hap 3	**24**	**26**		*11,06%*	*0,00%*	*10,21%*	*0,85%*	*10,64%*	*10,64%*	*11,06%*	*10,64%*	*10,64%*	*10,64%*	*11,06%*	*11,49%*	*11,06%*	*8,94%*	*9,36%*	*8,94%*	*9,36%*	*10,64%*	*11,06%*	*10,21%*	*11,06%*	*10,64%*	*10,64%*	*10,64%*
4. Hap 4	**2**	**6**	**26**		*11,06%*	*1,70%*	*11,06%*	*1,28%*	*2,13%*	*2,55%*	*10,21%*	*10,21%*	*9,79%*	*10,21%*	*10,64%*	*10,64%*	*7,23%*	*7,66%*	*7,23%*	*7,66%*	*2,13%*	*2,55%*	*1,70%*	*2,55%*	*2,13%*	*2,13%*	*2,13%*
5. Hap 5	**24**	**26**	**0**	**26**		*10,21%*	*0,85%*	*10,64%*	*10,64%*	*11,06%*	*10,64%*	*10,64%*	*10,64%*	*11,06%*	*11,49%*	*11,06%*	*8,94%*	*9,36%*	*8,94%*	*9,36%*	*10,64%*	*11,06%*	*10,21%*	*11,06%*	*10,64%*	*10,64%*	*10,64%*
6. Hap 6	**2**	**4**	**24**	**4**	**24**		*11,06%*	*1,28%*	*2,13%*	*2,55%*	*8,51%*	*8,51%*	*8,09%*	*8,51%*	*8,94%*	*8,94%*	*5,53%*	*5,96%*	*5,53%*	*5,96%*	*2,13%*	*1,70%*	*1,70%*	*2,55%*	*2,13%*	*2,13%*	*2,13%*
7. Hap 7	**24**	**26**	**2**	**26**	**2**	**26**		*10,64%*	*10,64%*	*11,06%*	*11,49%*	*11,49%*	*11,49%*	*11,91%*	*12,34%*	*11,91%*	*9,79%*	*10,21%*	*9,79%*	*10,21%*	*10,64%*	*11,06%*	*10,21%*	*11,06%*	*10,64%*	*10,64%*	*10,64%*
8. Hap 8	**1**	**5**	**25**	**3**	**25**	**3**	**25**		*1,70%*	*2,13%*	*9,79%*	*9,79%*	*9,36%*	*9,79%*	*10,21%*	*10,21%*	*6,81%*	*7,23%*	*6,81%*	*7,23%*	*1,70%*	*2,13%*	*1,28%*	*2,13%*	*1,70%*	*1,70%*	*1,70%*
9. Hap 9	**3**	**1**	**25**	**5**	**25**	**5**	**25**	**4**		*0,43%*	*8,09%*	*8,09%*	*7,66%*	*9,36%*	*9,36%*	*9,36%*	*5,11%*	*5,53%*	*5,11%*	*5,53%*	*0,00%*	*0,43%*	*0,43%*	*0,43%*	*0,00%*	*0,00%*	*0,00%*
10. Hap 10	**4**	**2**	**26**	**6**	**26**	**6**	**26**	**5**	**1**		*8,51%*	*8,51%*	*8,09%*	*9,79%*	*9,79%*	*9,79%*	*5,53%*	*5,96%*	*5,53%*	*5,96%*	*0,43%*	*0,85%*	*0,85%*	*0,85%*	*0,43%*	*0,43%*	*0,43%*
11. R. s. af Mozambique (JX195173)	**22**	**18**	**25**	**24**	**25**	**20**	**27**	**23**	**19**	**20**		*0,00%*	*0,43%*	*8,51%*	*8,51%*	*8,09%*	*4,68%*	*5,11%*	*4,68%*	*5,11%*	*8,09%*	*7,66%*	*8,51%*	*8,51%*	*8,09%*	*8,09%*	*8,09%*
12. R. s. af South Africa (GU553079)	**22**	**18**	**25**	**24**	**25**	**20**	**27**	**23**	**19**	**20**	**0**		*0,43%*	*8,51%*	*8,51%*	*8,09%*	*4,68%*	*5,11%*	*4,68%*	*5,11%*	*8,09%*	*7,66%*	*8,51%*	*8,51%*	*8,09%*	*8,09%*	*8,09%*
13. R. s. Brazil (JX2066980)	**21**	**17**	**25**	**23**	**25**	**19**	**27**	**22**	**18**	**19**	**1**	**1**		*8,09%*	*8,09%*	*7,66%*	*4,26%*	*4,68%*	*4,26%*	*4,68%*	*7,66%*	*7,23%*	*8,09%*	*8,09%*	*7,66%*	*7,66%*	*7,66%*
14. R. tur Italy (KC243856)	**22**	**21**	**26**	**24**	**26**	**20**	**28**	**23**	**22**	**23**	**20**	**20**	**19**		*0,85%*	*0,85%*	*5,53%*	*5,96%*	*5,53%*	*5,96%*	*9,36%*	*8,94%*	*8,94%*	*9,79%*	*9,36%*	*9,36%*	*9,36%*
15. R. tur Italy (KC243858)	**23**	**21**	**27**	**25**	**27**	**21**	**29**	**24**	**22**	**23**	**20**	**20**	**19**	**2**		*0,85%*	*5,53%*	*5,96%*	*5,53%*	*5,96%*	*9,36%*	*8,94%*	*8,94%*	*9,79%*	*9,36%*	*9,36%*	*9,36%*
16. R. tur Italy (KC243860)	**23**	**21**	**26**	**25**	**26**	**21**	**28**	**24**	**22**	**23**	**19**	**19**	**18**	**2**	**2**		*5,53%*	*5,96%*	*5,53%*	*5,96%*	*9,36%*	*8,94%*	*8,94%*	*9,79%*	*9,36%*	*9,36%*	*9,36%*
17. R. s. T1 Greece (KC243841)	**15**	**11**	**21**	**17**	**21**	**13**	**23**	**16**	**12**	**13**	**11**	**11**	**10**	**13**	**13**	**13**		*0,43%*	*0,00%*	*0,43%*	*5,11%*	*4,68%*	*5,53%*	*5,53%*	*5,11%*	*5,11%*	*5,11%*
18. R. s. T1 Greece (KC243840)	**16**	**12**	**22**	**18**	**22**	**14**	**24**	**17**	**13**	**14**	**12**	**12**	**11**	**14**	**14**	**14**	**1**		*0,43%*	*0,85%*	*5,53%*	*5,11%*	*5,96%*	*5,96%*	*5,53%*	*5,53%*	*5,53%*
19. R. s. T1 Greece (KC243842)	**15**	**11**	**21**	**17**	**21**	**13**	**23**	**16**	**12**	**13**	**11**	**11**	**10**	**13**	**13**	**13**	**0**	**1**		*0,43%*	*5,11%*	*4,68%*	*5,53%*	*5,53%*	*5,11%*	*5,11%*	*5,11%*
20. R. s. T1 Greece (KC243839)	**16**	**12**	**22**	**18**	**22**	**14**	**24**	**17**	**13**	**14**	**12**	**12**	**11**	**14**	**14**	**14**	**1**	**2**	**1**		*5,53%*	*5,11%*	*5,96%*	*5,96%*	*5,53%*	*5,53%*	*5,53%*
21. R. s. T2 Portugal (KC243844)	**3**	**1**	**25**	**5**	**25**	**5**	**25**	**4**	**0**	**1**	**19**	**19**	**18**	**22**	**22**	**22**	**12**	**13**	**12**	**13**		*0,43%*	*0,43%*	*0,43%*	*0,00%*	*0,00%*	*0,00%*
22. R. s. T2 Portugal (KC243845)	**4**	**0**	**26**	**6**	**26**	**4**	**26**	**5**	**1**	**2**	**18**	**18**	**17**	**21**	**21**	**21**	**11**	**12**	**11**	**12**	**1**		*0,85%*	*0,85%*	*0,43%*	*0,43%*	*0,43%*
23. R. s. T2Portugal (KC243846)	**2**	**2**	**24**	**4**	**24**	**4**	**24**	**3**	**1**	**2**	**20**	**20**	**19**	**21**	**21**	**21**	**13**	**14**	**13**	**14**	**1**	**2**		*0,85%*	*0,43%*	*0,43%*	*0,43%*
24. R. s. France (JX304697)	**4**	**2**	**26**	**6**	**26**	**6**	**26**	**5**	**1**	**2**	**20**	**20**	**19**	**23**	**23**	**23**	**13**	**14**	**13**	**14**	**1**	**2**	**2**		*0,43%*	*0,43%*	*0,43%*
25. R. s. France (JX304686)	**3**	**1**	**25**	**5**	**25**	**5**	**25**	**4**	**0**	**1**	**19**	**19**	**18**	**22**	**22**	**22**	**12**	**13**	**12**	**13**	**0**	**1**	**1**	**1**		*0,00%*	*0,00%*
26. R. s. Spain (GU553081)	**3**	**1**	**25**	**5**	**25**	**5**	**25**	**4**	**0**	**1**	**19**	**19**	**18**	**22**	**22**	**22**	**12**	**13**	**12**	**13**	**0**	**1**	**1**	**1**	**0**		*0,00%*
27 .R. s. T2 Spain (KC243843)	**3**	**1**	**25**	**5**	**25**	**5**	**25**	**4**	**0**	**1**	**19**	**19**	**18**	**22**	**22**	**22**	**12**	**13**	**12**	**13**	**0**	**1**	**1**	**1**	**0**	**0**	

Note: Hap- Haplotype; (R. s.) *R. sanguineus*; (R.tur). *R. turanicus.*

Once this analysis was concluded, it was decided to analyze how the haplotypes obtained in our study would relate in the phylogenetic point of view with sequences isolated from other geographical origins, in order to achieve that the neighbor-joining trees, figs. 49 and 50 were created.

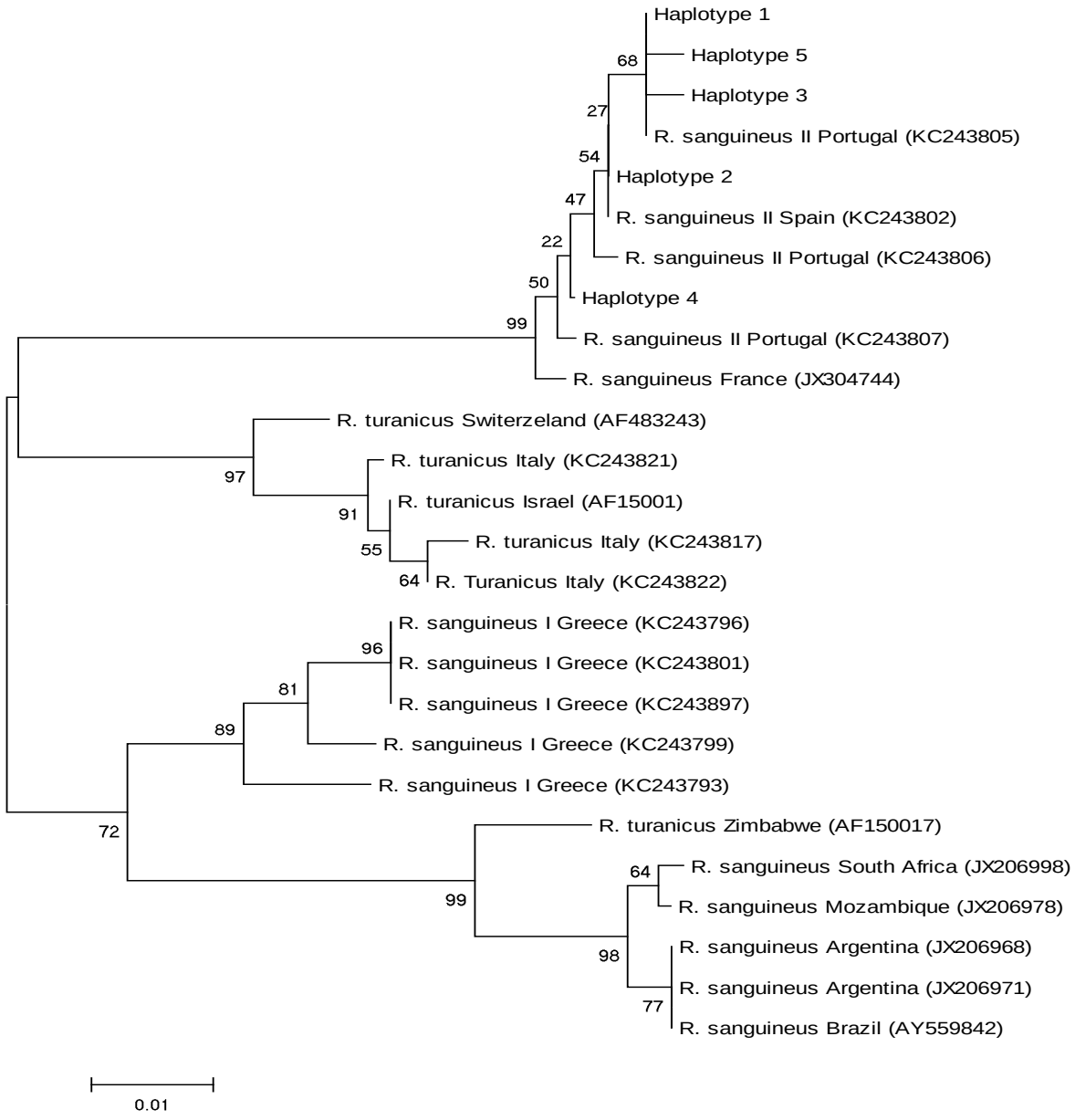


Fig. 49: Phylogeny of Rhipicephallus spp. Inferred from 12S rDNA.- Nieghbor-joining tree of the 12S rDNA gene using Tamura-Nei model. Haplotype, geographical origin and GenBank AC from each haplotype are presented between brackets. Bootstrap values are also presented in each bracket and are based on 1000 replicates.

By looking at fig.49, it is possible to observe the formation of four distinct groups It is also possible to conclude that all 5 haplotypes acquired in this study are exclusively grouped in the group *R. sanguineus* T2, alongside with the Portuguese (KC243805), (KC243806) and (KC243807), from Portugal, (KC243802) and (JX304744) sequences. One expected result, given the p-distance values displayed by Table 12, the fact that the 5 haplotypes, obtained in this study with the 12S marker, aren't presented in the same bracket, supports the results presented by table 5, suggesting the presence of some intra-specific variation, however not enough to require the classification as different species. The use of phylogenetic analysis also allows observing that, the closest group to the *R. sanguineus* T2 is the group *R. turanicus* formed by the sequences (KC243821), (KC243817) and (KC243822) isolated in Italy, (AF15001) and (AF483243) isolated respectively in Israel and Switzerland. The second closest group to *R. sanguineus* T2 is *R. sanguineus* T1, constituted by the sequences (KC243796), (KC243801), (KC243897), (KC243799) and (KC243793) all isolated in Greece. And finally the more distant group is the *R. sanguineus sensu lato* also described as the northern linage, constituted by the sequences (JX206998), isolated in South Africa, (JX206978) in Mozambique, (AY559842) in Brazil, (AF150017) in Zimbabwe and (JX206971) and (JX206971) in Argentina.

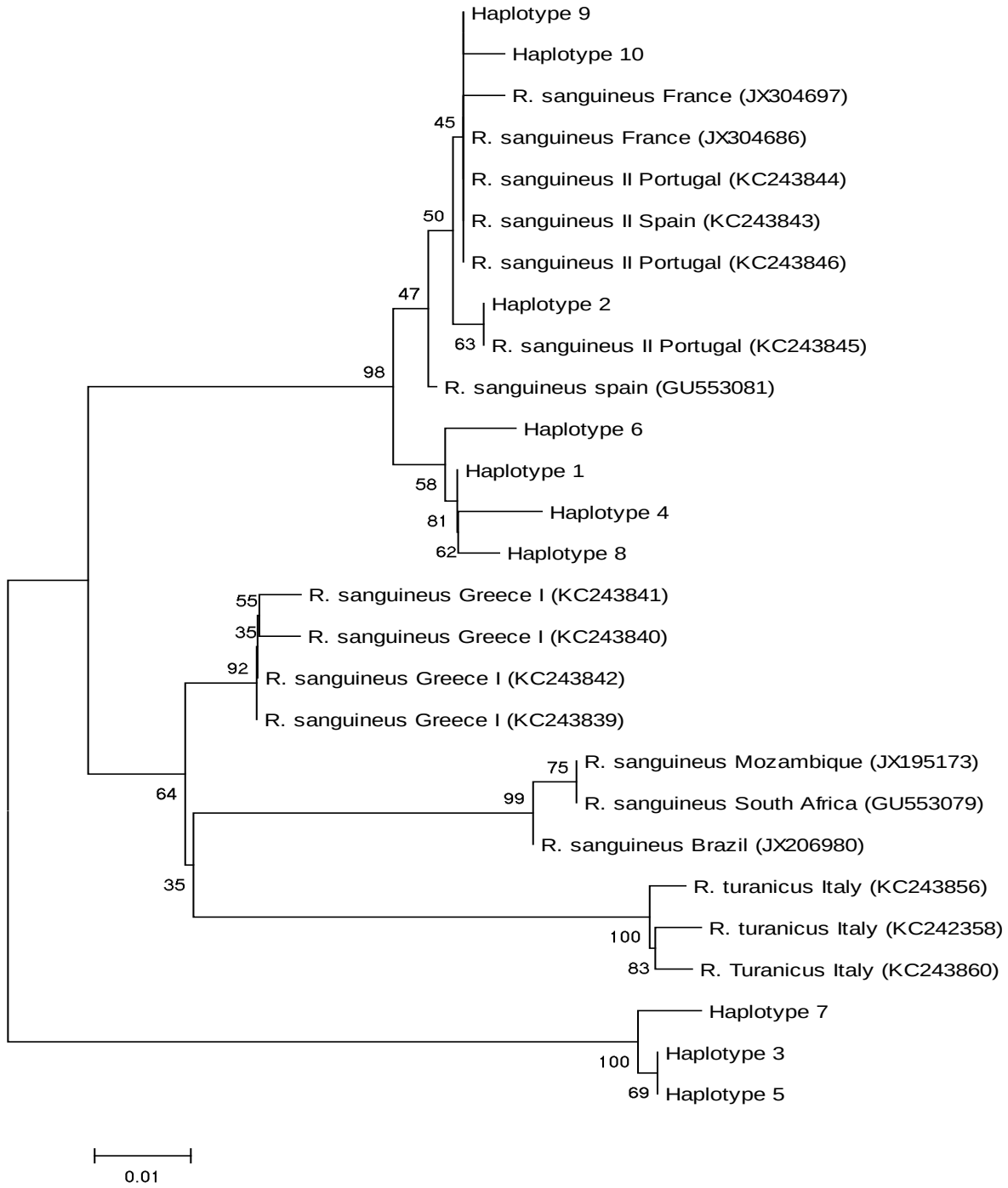


Fig. 50: Phylogeny of Rhipicephallus spp. Inferred from 16S rDNA.- Nieghbor-joining tree of the 16S rDNA gene using Tamura-Nei model. Haplotype, geographical origin and Genebank AC from each haplotype are presented between brackets. Bootstrap values are also presented in each bracket and are based on 1000 replicates.

In figure 50, it is possible to observe the formation of five distinct groups, where all haplotypes acquired in this study, excluding haplotypes 3, 5 and 7, that were include in this study as a control group, are exclusively gathered in the group *R. sanguineus* T2, alongside with the sequences (KC243844), (KC243846) and (KC243845), from Portugal, (KC243843)

and (GU553081) from Spain, (JX304697) and (JX304686) from France. This is an expected result, given the p-distance values displayed by Table 13. However, the haplotypes 6, 1, 4 e 8, despite the fact that aren't associated with p-distance values that justify the classification as a different species, these haplotypes are grouped in a separate tree branch, forming a kind of mini-clade. The use of phylogenetic analysis also allows observing that the closest group to the *R. sanguineus* T2 is the group *R. sanguineus* T1 formed by the sequences (KC243841), (KC243840), (KC243842) and (KC243839) all isolated in Greece. The second closest group to *R. sanguineus* T2 is *R. sanguineus sensu lato* also know as northern linage, composed by the sequences (GU553079), isolated in South Africa, (JX195173) in Mozambique, and (JX206980) in Brazil. At last the more distant group to *R. sanguineus* T2, if we exclude the control group, is the *R. turanicus* group, formed from the sequences (KC243856), (KC243858) and (KC243860) all with origin in Italy. To note that there is a fifth group, formed by the haplotypes 3, 5 and 7, that is farthest group of all, what is expected as a control-group.

With the purpose of studying the differences in the performance of the molecular markers, 12S and 16S Tables 14 and 15 were created.

Table 14 – Information of each element of the sample from which a sequence was isolated, using the 12S marker. Relative to the morphological cluster to which it belongs as well as the Blast result, identification certainly, AC, alongside with the sexual gender and the identifying numbers of each specimen.

INS	S	MC	AC	Gene	Ident.(%)
1594	M	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2118	M	D	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	99
1838	M	Tur	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1637	M	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2130	M	R	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1641	M	Af	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2140	M	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1869	M	Pus	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2380	M	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1957	M	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2405	M	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1959	M	D	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1693	M	Af	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	99
1629	M	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1992	M	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1755	M	Pus	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2159	M	Tur	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2086	M	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1807	M	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2111	M	Tur	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	99
2385	M	Tur	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1756	F	Tur	JX304709.1	Rhipicephalus sanguineusisolate dog#1.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1841	F	Tur	JX304709.1	Rhipicephalus sanguineusisolate dog#1.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1845	F	Tur	JX304709.1	Rhipicephalus sanguineusisolate dog#1.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1846	F	Tur	JX304731.1	Rhipicephalus sanguineusisolate dog#60.1 12S ribosomal RNA gene, partial sequence; mitochondrial	99
1860	F	Pus	JX304709.1	Rhipicephalus sanguineusisolate dog#1.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1861	F	Pus	JX304710.1	Rhipicephalus sanguineusisolate dog#1.4 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1990	F	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1999	F	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2002	F	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2045	F	T1	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
2114	F	Int	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	99
1609	F	Af	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1634	F	Int	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1700	F	Int	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1712	F	Int	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1774	F	T2	JX304744.1	Rhipicephalus sanguineusisolate dog#78.1 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1851	F	T1	JX304710.1	Rhipicephalus sanguineusisolate dog#1.4 12S ribosomal RNA gene, partial sequence; mitochondrial	100
1891	F	T1	JX304710.1	Rhipicephalus sanguineusisolate dog#1.4 12S ribosomal RNA gene, partial sequence; mitochondrial	100

Note: INS- Identification Number of the Specimen, **S-**sexual gender, **Ident(%)-** Certainly of identificacion, **AC**- GenBank Acess code **MC-** Morphologic Cluster, **M**- Male, **F-**Female, **Tur-** R. Turanicus, **Pus**- R. Pusillus, **D**- *R. Sanguineus* D, **R**- *R. Sanguineus R*, **Af**- *R. Sanguineus s. s.*, **T1**- *R. Sanguineus* Type 1, **T2**- *R. Sanguineus* Type 2, **Int**- *R. Sanguineus* Intermediate.

Table 15 – Information of each element of the sample from which a sequence was isolated, using the 16S marker. Relative to the morfological cluster to which it belongs as well as the Blast result, identification certainly, AC, alongside with the sexual gender and the identifying numbers of each specimen.

INS	S	MC	AC	Gene	Ident.(%)
1834	M	Tur	JX304685.1	Rhipicephalus sanguineus isolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
1838	M	Tur	JQ362399.1	Rhipicephalus sanguineusisolate dog#1.1 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1869	M	Pus	AJ002957.1	Rhipicephalus pusillus 16S mitochondrial rRNA gene, partial	98
1671	M	T1	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1957	M	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1959	M	D	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2064	M	Tur	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2086	M	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
2111	M	Tur	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
2118	M	D	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2130	M	R	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2380	M	T1	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1629	M	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1755	M	Pus	AJ002957.1	Rhipicephalus pusillus 16S mitochondrial rRNA gene, partial	99
2159	M	Tur	JQ362399.1	Rhipicephalus sanguineusisolate dog#1.1 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2242	M	Tur	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2385	M	Tur	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
1610	F	Tur	AJ002957.1	Rhipicephalus pusillus 16S mitochondrial rRNA gene, partial	98
1841	F	Tur	JQ362399.1	Rhipicephalus sanguineusisolate dog#1.1 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1845	F	Tur	JQ362399.1	Rhipicephalus sanguineusisolate dog#1.1 16S ribosomal RNA gene, partial sequence; mitochondrial	99
1846	F	Tur	JQ362399.1	Rhipicephalus sanguineusisolate dog#1.1 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1860	F	Pus	AJ002957.1	Rhipicephalus pusillus 16S mitochondrial rRNA gene, partial	98
1990	F	T1	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
1999	F	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
2002	F	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
2045	F	T1	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
2114	F	Int	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1609	F	Af	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1634	F	Int	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	99
1700	F	Int	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1712	F	Int	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1774	F	T2	JX304685.1	Rhipicephalus sanguineusisolate dog#18.2 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1848	F	T2	JQ362400.1	Rhipicephalus sanguineusisolate dog#1.4 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1851	F	T1	JQ362400.1	Rhipicephalus sanguineusisolate dog#1.4 16S ribosomal RNA gene, partial sequence; mitochondrial	100
1891	F	T1	JQ362400.1	Rhipicephalus sanguineusisolate dog#1.4 16S ribosomal RNA gene, partial sequence; mitochondrial	99

Note: INS- Identification Number of the Specimen, **S**-sexual gender, **Ident(%)**- Certainly of identificacion, , **AC**- GenBank Acess code **MC-** Morphologic Cluster, **M**- Male, **F**-Female, **Tur**- R. Turanicus, **Pus-** *R. Pusillus*, **D**- *R. Sanguineus* D, **R**- *R. Sanguineus R*, **Af-** *R. Sanguineus s .s*, **T1**- *R. Sanguineus* Type 1, **T2**- *R. Sanguineus* Type 2, **Int**- *R. Sanguineus* Intermediate.

Final remarks:

At this point, it is possible to note that the marker 12S allowed to acquire more sequences in optimal conditions, than the marker 16S, (39 vs 34). Despite that fact, it is possible to conclude that, in this study, the marker 16S showed more discrimination power, since 12S marker only allowed to obtain five haplotypes, while 16S allowed to obtain ten haplotypes and, simultaneously, the p-distances values associated with the haplotypes acquired with 12S

ranged from, 0,28% to 0,83%, whereas the p-distances associated with the haplotypes acquired with 16S, varried from 0,43% to 1,71%. Both values indicate intra-specific variation; however the values associated with the 16S molecular marker point to a higher level of intra-specific variation. Nonetheless, the main evidence that the 16S marker showed more characterization power is the fact that 12S failed to identify the specimens that were added to the study as control group, *R. pusillus*, while marker 16S correctly identified them, allowing the appearance of the haplotypes 3, 5 and 7.

The overall p-distances values ranged from 0,28% to 1,71%, suggesting some interesting levels of intra-specific variation, however, not enough to justify the classification as different species. The data presented by the tables 12 and 13, and figs 49 and 50, indicate that all haplotypes obtained in this study are phylogenetically close to the sequences isolated, in specimens with origin in France, Spain and in previous studies conducted in Portugal, as evidenced by the p-distance values and the fact that in both neighbor joining trees, all haplotypes acquired in this study grouped with the sequences isolated in those countries, in the *R. sanguineus* T2 group. An expected result if we consider that these 3 countries have in common plenty of geo-environmental characteristics.

By observing both trees, created to infer the phylogeny of the genes 12S and 16S, in the tree associated to the 12S gene, the closest group to *R. sanguineus* T2 is the *R. turanicus* group constituted by sequences isolated in Italy, Switzerland, and Israel, followed by the group *R. sanguineus* T1 formed with sequences isolated in Greece, and the phylogenetically more distant group is the one constituted by sequences isolated in tropical countries, also known as *R. sanguineus sensu lato* group or northern linage. In turn the tree associated to the 16S gene presents a similar structure but with, several differences, namely, the closest group to the *R. sanguineus* T2 group is the *R. sanguineus* T1 formed with sequences isolated in Greece and the farthest group, apart from the haplotypes added to this study to function as a control-group, is the *R. turanicus* group, constituted with sequences isolated in Italy.

It is also noteworthy that the haplotypes 6, 1, 4 e 8 acquired with the 16S marker, in spite of the fact that aren't associated with p-distance values that justify the classification as a different species, when a phylogenetic analysis is performed, it is possible to state that these haplotypes are grouped in a separate tree branch, forming a kind of mini-clade. Suggesting that it is possible that what it´s being observed is the beginning of a speciation process, as

such, there isn't enough genetic variability that supports the distinction as different species at the present time, but in the future, that might occur, once there is already enough intra-variability, that leads some haplotypes to group in a separated brunch.

One evidence that supports this hypothesis is the fact the different haplotypes obtained in this study, at the moment of the morphologic classification, were classified as different morphologic groups, namely *R. turanicus, R. pusillus, R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* s. l., and *R. sanguineus* Intermediate. So despite the fact that the genetic differences aren't pronounced enough yet to justify the classification as different species, the morphologic differences already validate that classification and it is possible that in the future, the genetic differences could increase.

5. Discussion

It was previously stated that ticks from the *R. sanguineus* group are historically associated with one of the groups around which there is less consensus, finding itself surrounded by controversy. Mainly considering that the identification and distinction of two species *R. sanguineus* and *R. turanicus* is a particularly challenging task even for experienced morphologists [15]. This controversy is the result of several factors namely, the fact that the original description of *Rhipicephalus sanguineus* provided by Latreille [102] lacked detail, what was acceptable for that time, when tick taxonomy was taking its first steps, but not for present time standards. It falls short of what would be acceptable as a proper description [32]. Associated with this problem, other questions arise, which further aggravates this problem such as: the type-specimen of *R. sanguineus* has been lost and little is known of its origin and *R. turanicus* description is quite similar to *R. sanguineus*. Thus the lack of morphological features to clearly distinguish both species, moreover in some geographic locations, these species are found in sympatry, and ticks of a given location might evidence slightly variations in some of their morphological features, under the same genetic background [3, 15, 18, 30]. Simultaneously, it was reported species *R. sanguineus* is ecologically flexible and tolerant to a wide variety of climatic conditions. It has the ability of suffer different selective pressures and develop different adaptation strategies, so it can be supposed, that the wide geographical distribution of this species, led to the appearance of subpopulations with distinct features [12]. Before such evidence is undeniable that, from an ecological point of view, *R. sanguineus* is a polymorphic species and in turn *R. turanicus* is considered a more limited species [3, 12]. However, in spite of those limitations, it was proved that *R. turanicus* is also a polymorphic species, once it was found considerable morphological differences between African populations and populations with origin in Cyprus, despite the fact that were genetically compatible [15]. In addition to all these factors that contribute to the controversy surrounding the *R. sanguineus* group, especially concerning *R. sanguineus s. l.* and *R. turanicus*, different species may potentially mate in the field and the existence of hybrids among field-collected tick specimens cannot be ruled out, mainly in areas where close related species occur together (sympatrically) [32].

Given that, some ticks belonging to genus *Rhipicephalus* are extremely difficult to identify morphologically, due to the high level of intraspecific variability, so morphological studies

should be applied together with biological and molecular studies to promote more consistent taxonomic reconstructions, it is in this context that this study appears. Once it combined an extensive morphological study, in which several quantitative and qualitative variables were considered and studied by a statistic analysis as well as by a rigorous morphological analysis that result in the formation of morphological, qualitative and quantitative clusters. From here it was possible to evaluate the differences between obtained clusters, and highlight the variables that most contribute to their differentiation, in both male and female samples, which lead to the conclusion of a wide morphological variety. The next logical step was to understand if this morphological variety also corresponded to genetic diversity. In order to achieve that goal, several specimens representing the various formed clusters were selected for a genetic study using the 12S and 16S molecular marker.

The findings obtained in this study regarding the morphologic and statistical analysis can be integrated in the following context:

R. sanguineus from the morphological point of view is very similar to *R. turanicus*, despite that there are several morphological structures, that can be used as a tool to differentiate both species, namely by examining the females genital aperture and the ending tail of the male spiracle [31].

However it is believed that the most differentiating morphological traits for this two species are the adanal plates for males, the genital aperture for female, and the spiracular plates for both genders [17]. Posteriorly it was claimed that the intraspecific morphological variations among ticks identified as *R. sanguineus* and *R. turanicus* were evident mainly in terms of colour, size, scutal punctuation pattern, female genital opening shape, spiracular plate shape, male adanal plate shape and male caudal process [32].

Although different authors defend distinct opinions about how to differentiate this two species, the best morphological differences to distinguish *R. sanguineus* and *R. turanicus* are: in males, if the tail ending of the spiracle is lesser or equal to half of the adjacent festoon, we are in the presence of a *R. sanguineus*, if it is greater than half of the adjacent festoon, *R. turanicus*, are the species involved; in females, *R sanguineus* presents a genital opening in the shape of a broad and open U with sclerites slightly wider than higher and far apart from each other; on the other hand *R. turanicus* shows a genital opening in the shape of a close U, with sclerites higher than wider and closer to each other; the ending of the spiracular tail is higher

and narrower in *R. sanguineus* while in *R. turanicus* this structure is wider and shorter [15, 34].

Simultaneously, it was described the existence of 4 morphologic groups; *R. sanguineus* I to IV, showing morphological characters distict from known species, but closely related to *R. turanicus* and to *R. sanguineus sensu lato*, in terms of the punctuation pattern on dorsal scutum in females, shape of the adanal plates and the accessory shields in males, and the shape of spiracular plates in both genders [32].

Considering these data, morphologic and statistical analysis were performed considering a series of quantitative and qualitative variables, in order to understand the morphologic diversity of the Portuguese populations of *R. sanguineus* and also analyze witch morphological features are the more adequate to distinguish *R. sanguineus* from *R. turanicus* and from the intermediate forms described in this study.

Results demonstrated that there is a lot of morphological diversity in the Portuguese populations of *R. sanguineus* once that morphological analyses reveled the formation of 8 morphologic clusters in males namely: *R. sanguineus sensu stricto*, *R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* D, *R. sanguineus* R, *R. turanicus*, *R. turanicus* D, and *R. pusillus* and also the presence of 5 morphologic cluster in females namely, *R. sanguineus sensu stricto*, *R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* Intermediate, *R. turanicus* and *R. pusillus*. These morphologic clusters differ from each other in terms of several morphological structures especially at the spiracular plate and at the genital region in females.

The statistical analyses lead to the formation of three quantitative clusters and three qualitative clusters, this situation occurred with both male and female data. It is also observable that these clusters are associated and related with the morphologic clusters in a very distinctive form that reflect what morphologic features define each morphologic group.

When analyzing the males quantitative and qualitative clusters it was observed that:

- Quantitative cluster 1 presents in its constitution elements belonging to the following morphologic clusters: *R. sanguineus* D, *R. sanguineus* T1, *R. sanguineus* T2 and *R. sanguineus* s. s. showing a stronger association with the morphologic clusters *R. sanguineus*,

R. sanguineus T1. These associations come as a logic result once that this morphologic clusters present elements that are associated with the narrowest spiracles of the sample, displaying the thinner ending of the spiracle tail in relation to the adjacent festoon.

- Quantitative Cluster 2 includes elements belonging to the following morphologic clusters: *R. sanguineus* s. s. *R. sanguineus* T1, *R. sanguineus* T2 *R. sanguineus* D, *R. sanguineus* R and *R. turanicus*. Data show the existence of an association between this quantitative cluster and morphologic clusters, *R. sanguineus* T1 and *R. sanguineus* T2, which reflects a natural result once both morphologic clusters represent intermediate forms and present elements that are associated with spiracles that present an intermediate form between *R. sanguineus* and *R. turanicus*.

- Quantitative Cluster 3 is composed by elements belonging to the following morphologic clusters: *R. sanguineus* T1, *R. sanguineus* T2, *R. turanicus, R. turanicus* D, *R. pusillus,* showing a stronger association with the morphologic clusters *R. turanicus* and *R. pusillus*. That is also something expected once this morphologic clusters present elements that are related with the wider spiracles of the sample, displaying the larger endings of the tail in relation to the adjacent festoon.

- Qualitative Cluster 1 contains elements belonging to the following morphologic clusters: *R. sanguineus s. s.*, *R. sanguineus* T1, *R. sanguineus* T2 *R. sanguineus* D, *R. sanguineus* R, *R.pusillus, R. turanicus* and *R. Turanicus* D. Data show the existence of an association between this quantitative cluster and morphologic clusters *R. sanguineus* T1 and *R. sanguineus* T2, which are the most represented morphologic groups within this cluster.

- Qualitative Cluster 2 comprises elements belonging to the following morphologic clusters: *R. sanguineus s. s., R. sanguineus* T1, *R. sanguineus* T2 *R. sanguineus* D, *R. sanguineus* R and *R. turanicus*. Data show the existence of an association between this quantitative cluster and morphologic clusters *R. sanguineus* T1 and *R. sanguineus* T2, which are the most represented morphologic groups within this cluster.

- Qualitative Cluster 3 exhibits elements belonging to the following morphologic clusters: *R. sanguineus s. s., R. sanguineus* T1, *R. sanguineus* T2 *R. sanguineus* D, *R. Pusillus, R. Turanicus* D and *R. turanicus*. Data show the existence of an association between this

quantitative cluster and morphologic clusters *R. sanguineus* T1 and *R. sanguineus* T2, a similar situation to what was observed in the two others qualitative clusters. However this time there is also the presence of an association with the morphologic cluster *R. pusillus*

It is possible to establish an association between each quantitative clusters and morphologic groups, however that association did not occur with the qualitative clusters, once that after analyzing the quanlitative clusters individually, it is possible to infer that there are certain characteristics common to all; in particular they all feature between 6 to 8 morphological groups. The presence of so many morphological groups suggests that the all qualitative clusters formed in this study have large intra-specific variation, and therefore it is difficult to characterize the morphological features that define them and as well as those that differentiate them. Simultaneously is also observable that each of the qualitative clusters features the two largest morphological clusters of the sample considered, *R. sanguineus* T1 and *R. sanguineus* T2, as the most representatives in its constitution; the unique exception is the association establish between the morphologic cluster *R. pusillus* and the qualitative cluster 3. Latter association occurs because the species *R. pusillus* contains several morphological features, which differ from those of other morphological groups, presented in this study, namely shorter dimensions, a distinctive spiracular area and their conscutums present a regular pattern of punctuation distribution. Taking this into account, probably the qualitative variables, "Spiracular area type", "Conscutum punctuation size" and "Conscutum punctuation distribution" were the ones that gave the bigger contribute to the insertion of *R. pusillus* specimens in the qualitative cluster 3, what results in a strong presence of the elements that belong to that species within that cluster. Apart from what happens in qualitative cluster 3 regarding *R. pusillus*, every qualitative cluster present a morphologic distribution, resembling the one presented by the total sample where the less numerous morphological groups such as *R. sanguineus* D, *R. sanguineus s. s.*, *R. turanicus* D and *R. pusillus* are represented with very few elements, morphological groups with an intermediate expression as *R. sanguineus* and *R. turanicus* have a reasonable expressiveness and the most expressive elements of sample *R. sanguineus* T1 and *R. sanguineus* T2, emerge as the dominant elements.

These facts suggest that there isn't really a clear and strong association between a morphological cluster and a qualitative cluster, once the various morphological groups are inserted into the three qualitative clusters in identical proportions to those presented by the total sample considered for this study. This indicates that the qualitative variables and,

consequently, the qualitative clusters have less differentiation capacity and less characterization power than the quantitative clusters.

On the other hand, each one of the quantitative clusters formed in this study presents fewer morphological groups, implying the establishment of a more specific association between this type of clusters and the morphological clusters. Simultaneously, the associations formed between the various morphologic and quantitative clusters, namely *R. sanguineus* T1 and *R. sanguineus* af with quantitative cluster 1, *R. turanicus* and *R. pusillus* with the cluster 3 and *R. sanguineus* T1 and *R. sanguineus* T2 with the quantitative cluster 2, suggest that the quantitative clusters have more differentiation capacity and more characterization power than the quantitative clusters

When performing a similar analysis to the female's quantitative and qualitative clusters it is possible to observe that:

- Quantitative cluster 1 presents in its constitution elements belonging to the following morphologic clusters: *R. sanguineus s. s.*, *R. sanguineus* Intermediate, *R. sanguineus* T2 and some outliers from *R. sanguineus* T1 and *R. pusillus*, showing the strongest association with the morphologic clusters *R. sanguineus* T2, but also associations with *R. sanguineus s. s.*, and *R. sanguineus* Intermediate. That is a natural result once all these morphologic clusters show elements that are associated with spiracles presenting large angles and higher and thin tails, large spiracles, and wide genital pore apertures.

- Quantitative Cluster 2 shows in its composition elements belonging to the following morphologic clusters: *R. sanguineus s. s.*, *R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* Intermediate, *R. pusillus* and *R. turanicus*. The existence of an association between this quantitative cluster and morphologic clusters *R. pusillus* and *R.turanicus* is an expected result, once both morphologic clusters evidence elements that are relaed with spiracles that present small angles and short tails with large width and the narrowest genital pore aperture of the sample.

- Quantitative Cluster 3 consists in elements belonging to the following morphologic clusters: *R. sanguineus* T1, *R. sanguineus* T2 and *R. sanguineus s. s.* showing a stronger association with the morphologic clusters *R. sanguineus* T1 and also a weaker association with *R.*

sanguineus T2, which can be considered normal result once both morphologic clusters show elements that are connect to specimens with spiracles that present higher tails, large angles, and wide genital pore apertures and both represent intermediate forms.

- Qualitative cluster 1 includes elements from the following morphologic clusters: *R. sanguineus s. s.*, *R. pusillus* and *R. sanguineus* T1 showing the strongest association with the morphologic cluster *R. pusillus*, but also associations with *R. sanguineus s. l.*, and *R. sanguineus* T1, which is composed by elements that are characterized by the pattern 1 genital aperture type in the case of *R. pusillus*, and with the pattern 2 type in the case of both *R. sanguineus*, *s. l.* and *R. sanguineus* T1, so this associations come out as natural result once that the pattern 1 is the most similar to the pattern 2.

- Qualitative Cluster 2 comprises elements included in the following morphologic clusters: *R. sanguineus s. s.*, *R. sanguineus* T1, *R. sanguineus* T2 *R. sanguineus* Intermediate and *R. turanicus*. Data show the existence of a very strong association between this quantitative cluster and morphologic clusters *R. sanguineus* T2 and also the presence of weaker associations with the morphologic groups, *R. turanicus* and *R. sanguineus* Intermediate. The main characteriscs exihibited by these elements are genital apertures whose feature the pattern 4 and 5 respectively, *R. sanguineus* T2 presents the pattern 3.

- Qualitative Cluster 3 presents in its constitution elements belonging to the following morphologic clusters: *R. sanguineus s. s. R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* Intermediate, *R. pusillus* and *R. turanicus*, showing an association between this quantitative cluster and morphologic clusters *R. sanguineus* T2, however weaker than the one registered in the Qualitative Cluster 2.

At this point it is possible to compare, not only the results of qualitative variables with the ones from the quantitative variables, but also compare the results obtained with the male and female sample.

So it is possible to conclude that in both cases the quantitative clusters have more differentiation capacity and more characterization power than the quantitative clusters, just like what was observed in males. Despite that, the qualitative clusters have a very different performance, in the female sample than what it was seen in the male one. Though, when

applied to the male sample, the qualitative clusters did not establish a clear and strong association with the morphological clusters, except for the morphologic cluster *R. pusillus* and the qualitative cluster 3. As a result, the various morphological groups are inserted into the three qualitative clusters in identical proportions to those obtained by the total sample considered in this study. By the opposite, when applied to the female sample, clear associations with morphologic clusters are revealed, such as the ones established between qualitative cluster 1 and *R. pusillus*, *R. sanguineus* s. s. and *R. sanguineus* T1, and also between qualitative cluster 2 and *R. turanicus*, *R. sanguineus* Intermediate and *R. sanguineus* T2. In fact, the only cluster that revealed weaker differentiation capacity was qualitative cluster 3, especially, because it does not evidenced a strong association like those between qualitative cluster 1 and *R. pusillus* and qualitative cluster 2 and *R. sanguineus* T2, nevertheless it is still able to show a weak association with morphologic cluster *R. sanguineus* T2.

So, the quantitative variables have a stronger performance than the qualitative variables, although in the female sample, they provide some interesting results. The fact that qualitative clusters in females have more differentiation capacity, than the qualitative clusters in males, is due to the presence of a very important variable "Genital aperture form", which reinforces the characterization power to the female quantitative clusters, allowing differentiation between the several morphologic clusters. In turn this is not verified in the male sample.

When comparing the results of this study to others described in the literature as to the most suitable morphologic characteristics to distinguish *R. sanguineus* from *R. turanicus* and from the intermediate forms, it is possible to conclude that there are some points of agreements namely the literature refers to the spiracular plate as one of the main morphological characteristic in males, the results obtained confirm this theory, once that all quantitative variables studied, "Spiracular tail final width/adjacent festoon final width ratio", "Spiracular oval area height/width ratio" "Spiracular third-widths ratio" and "Spiracular area tail angle", were those that contributed the most to the quantitative cluster formation.

In fact, literature claims that if the termination of the tail of the spiracle is lesser or equal to half of the adjacent festoon, we are in the presence of a *R. sanguineus*, when we are in the presence of a *R. turanicus*, the termination of the tail of the spiracle is larger than half of the adjacent festoon [15].

Those were witnessed in the results obtained in this study once that *R. sanguineus sensu lato* shows 0,367 millimeters and *R. turanicus* presented 0,677 millimeters in concern of the variable "Spiracular tail final width/adjacent festoon final width ratio" furthermore this variable revealed itself as the most important one in the male statistical analysis.

Concerning males specimens, the comparison of the obtained results in this study with others described in literature, the main difference is that the adanal plates are often described as one important distinguishing feature [17, 78]. However in our study that was not evidenced since the quantitative variable "Adanal plates height/width ratio" did not contribute to the quantitative clusters formation. In turn, the qualitative variable "Adanal plates ending" had a moderate statistical significant effect on the quantitative clusters formation, but even considering this, the Adanal plates, failed as a distinguishing morphologic feature. This results can be explained by the eventually hibridation between *R. sanguineus* and *R. turanicus* in the field, whose intermediated adanal plates could hardly be used as an effective separation criteria [33]. Different species may potentially mate in the field and the existence of hybrids among field-collected tick specimens cannot be ruled out [32]. When looking at the composition of this male sample hibridation is a possibility since 71,5% where morphologically classified as Intermediate forms, namely *R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* D and *R. sanguineus* R.

Applying the same comparison to females the results evidenced are in agreement with those usually stated by the literature pointing out the female genital aperture, the spiracular plate [15, 17] and the dorsal scutum [32], as the most importante criteria. In fact all quantitative variables associated with the genital region of females contributed for the quantitative clusters formation. Similary all the variables regarding the spiracular region and the variable "Scutum Lenght/Width ratio" gave a relevant contribute to the quantitative clusters formation.

Literature also refers that the spiracular plate is not as relevant in females as in malespointing out the genital aperture as the major distinctive characteristic [15]. Our data also support this statement, as the quantitative variables that gave the most important contribute to the quantitative clusters formation were "Sclerites insertion ratio" and "Sclerites height/width ratio" followed by the variables "Genital pore aperture", all these three variables are related with the shape and measurements of the genital region. The quantitative variable associated to

spiracular areas, that is better positioned in terms of it´s contribution to cluster formation only appears in the fourth position. The same behavior occurs, when considering the qualitative variables, where "Genital aperture shape" was the one that gave the greatest contribution for the clusters formation.

In addition, literature mentioned that *R sanguineus* is characterized by a genital opening in the shape of an open U with sclerites far apart from each other; and on the other hand *R. turanicus* shows a genital opening in the shape of a close U, with sclerites closer to each other. These features were also confirmed by the results since the variable "Genital pore aperture" that measured the distance between the sclerites, show an average value of 73,032 millimeters for *R sanguineus s. s.* (africanus) and 24,569 millimeters for *R. turanicus.*

Regarding the genetic analysis, the following chain of events can be considered

In 1994 the mitochondrial marker 16S was used to infer the phylogeny of hard and soft ticks. The results largely supported the phylogeny derived so far but, simultaneous indicated some alterations, allowing to demonstrate that this marker was adequated to perform genetic studies in ticks. Addicionally, it also contributed for the understanding of the origin of Ixodidae, which in geo-chronological terms it shouldhave occurred somewhere in the late Cretaceous. The results also pointed out for genetic differences between *R. sanguineus* and *R. turanicus* was about at 5,7% [19].

In 1998 these results found support on another study which also inferred the phylogeny of ticks, using marker 16S, achieving the conclusion that *R. sanguineus* and *R. turanicus*, had recently diverged from the genus *Rhipicephalus*, based on the high percentage of similarity presented by the genetic sequences of each species. These study also provide further validation of this marker by concluding that it was quite suitable for species of ticks closely related but also useful for comparison of distantly related taxa [21]. These findings were supported and extend to the molecular marker 12S by several authors [20, 22].

Another phylogenic study, using the 12S mitochondrial marker, provide information that had not been previously considered in a phylogenic study and simultaneously, some interesting values regarding *R. sanguineus* and *R. turanicus* genetic distances. The differences between the sequences of *R. sanguineus* from the northwestern Mediterranean coast and the sequences isolated in Turkmenistan was only 2,4%. In turn, when *R. sanguineus* Mediterranean species

was compared with *R. turanicus* sequences isolated from South African specimens, the difference increases to values that range from 5,9% to 8,3%. A similar situation occurs when comparing French sequences with, sequences isolated in Zimbabwe, which showed 7,7% of distance. However, when comparing the same French sequences with others isolated in Greece the values ranged between 4,4% and 5,6%. Finally, it was suggested that divergence up to 7,8% indicates an intra-specific variation and higher values evidenced inter-specific variation [18].

The interest in the controversy regarding *R. sanguineus* and *R. turanicus* was intensified after relevant morphologic differences were found between *R. sanguineus* from Brazil and *R. sanguineus* from Argentina. The use of SEM in populations of *R. sanguineus* from both these countries reveled several morphologic differences, namely at female genital aperture. Though, it was noticed that females from Brazil showed a genital aperture of broad V-shape (a characteristic of *R. turanicus*) and females from Argentina evidenced a U-shaped genital aperture (characteristic of *R. sanguineus*). Such findings suggested the existence of at least two different populations in South America [84]. These data emerged as a sequence of previous studies that had already detected morphological variations in the adanal plates, spiracular plates, hypostomal dentition, genital aperture and palpi in *R. sanguineus* from eight states of Brazil [30]. However, until recently it was believed that *R. sanguineus sensu stricto* was the only representative of the genus in South America [3].

The hypothesis of two different populations in South America gained more support, when the existence of two very dissimilar populations in South America was demonstrated, namely, the populations of Rafaela, (Santa Fé, Argentina) and the population in Jaboticabal (S. Paulo, Brazil). It was concluded that this populations presented considerable genetic differences, once the absolute nucleotide difference was 27 and the p-distance between these populations was 8%. Simultaneously, it was possible to verify that the p-distance between the populations of *R. sanguineus* in Argentina and the population of *R. sanguineus* in France only ranged from 0% to 0,6%, while between the populations of *R. sanguineus* in Brazil and the populations of *R. sanguineus* in Israel it reached to 8,3%. In turn, when the population of *R. sanguineus* in Brazil was compared with *R. turanicus* from Zimbabwe the distances only attained 2,4%. Therefore it was possible to conclude that populations of *R. sanguineus* in Argentina were closely related to European populations of *R. sanguineus* and populations of *R. sanguineus* of Brazil were closely related to African populations. Moreover, it was demonstrated that these populations also presented differences in feeding and reproductive parameters, and that some

hybrid larvae obtained experimentally between the Brazilian stains and Argentine strains of *R. sanguineus* were infertile [85]. This fact contributed to support the separation of these two populations, considering that some authors suggest that the production of viable offspring is a species ability [103].

Posteriorly, the comparison between genetics strains from Brazilian ticks with origin in several regions was performed using the mitochondrial molecular markers, 12S and 16S. The p-distance among Brazilian samples ranged from 0,0% to 6,0% with the molecular 12S, and ranged from 0,0% to 2,7% with the molecular marker 16S. When comparing the Brazilian sequences with others from different countries, the dissimilarities ranged from 0,0% to 15,9% and 0,0% to 9,8% respectively. Considering the results presented by the gene 12S, an overall strong genetic relationship was detected between *R. sanguineus* from Brazil and Asia (Taiwan and Thailand) and also with *R. turanicus* from Africa (Zimbabwe and Zambia). On the other hand, populations of *R. sanguineus* from Argentina and Uruguay appear to be related to French, Egyptian and North American sequences. Similar results were presented by the 16S gene However some differences were noticed, namely the Brazilian sequences did not have such a marked distance between them as seen in the 12S gene, and his distance to some European sequences was higher. Also it was noted that some sequences classified as *R. turanicus*, when phylogenetic analysis was performed, appear to be closely related to *R. sanguineus*, a phenomenon that has already been observed in specimens from, Europe and South Africa[86].

The use of the 16S molecular marker with the purpose of comparing genetic sequences from several European and South American countries caused the formation of two clades: one formed with sequences from *R. sanguineus* and *R. turanicus*, with origin in Mexico, Costa Rica, Panama, Colombia, Venezuela and South Africa and another one formed by sequences from *R. sanguineus* and *R. turanicus* from Europe and also Chile, Argentina and Uruguay. The differences between the two clades, ranging between 5,85% and 6,96%, suggest the existence of two species, one associated with tropical climate and another one related to a more temperate climate with lower temperatures. The fact that the sequences included in the tropical clade, present p-distances that range from 1,39% to 1,95% when compared to African sequences, but in turn present p-distances that range from 5,01% to 5,57% when compared, to European sequences, supports this theory. The opposite occurs when the sequences from the Temperate clade are compared with European sequences, the p-distance ranged from 0,0%

to 0,28%, and when compared with African sequences the p-distance rages from 6,13% to 6,14% [87].

In the context of these results, an analysis of *R. sanguineus sensu lato* was performed in the Southern cone of South America. Thirteen different haplotypes were found separated in two groups by phylogenetic analysis that represented the southern linage and the northern linage. The southern linage is formed by haploytpes isolated in Argentina, Uruguay, Chile and Italy which is closely related to European sequences and is associated with temperate areas. The northern linage is composed by sequences isolated in Mozambique, Brazil, Paraguay, Colombia, South Africa, and in the North of Argentina, which is closely related to African sequences, and is associate with tropical climate. This study was performed using the molecular markers 12S and 16S, and it was evidenced by the p-distance among the haplotypes within each clusters ranged from 0,1% to 0,4%, using the 16S marker and the difference between the northern and the southern linage varied from 4,9% to 6,5%. Using the 12S marker, the p-distance among the haplotypes within each clusters ranged from 0,0% to 1,3% and the difference between the northern and the southern linage varried from 7,6% to 8,5% [88]. It t was also verified a pattern of distribution that forms a latitudinal gradient for each linage, which is consistent with the previous discussed results [86, 87].

An identical situation occurs in North America. The comparison of genetic strains of ticks using the molecular marker 12S revealed that the sequences from Oklahoma were closely related to sequences isolated in Israel and were significantly distant from the ones isolated in South Africa. In the same way, phylogenic analysis inferred with this gene revealed that the sequences of *R. sanguineus* from Los Angeles, Atlanta and Arizona are closely related to sequences isolated from Rafaela, Argentina and in turn, sequences of *R. sanguineus* from Saint Kitts, are closely related to the tropical clade. Sequences isolated in Colorado and Oklahoma are related with the temperate clade, but present p-distances that range from 3% to 3,5% when compared with the ones isolated in Rafaela. It was also noticed that some sequences with origin in different countries identified as *R. sanguineus* appear to be related with clades mainly formed by *R. turanicus* [10].

The next step was to sequence the full mitochondrial genome, which was performed on *R. sanguineus* from China and on *R. sanguineus* from USA. For the 13 protein-coding genes comparisons revealed divergences that ranged between 9,34% and 15,65%. In addition sequence comparison of the genes cox 1 and CYTB among *R. sanguineus* was also performed

showing substantial nucleotide difference between the populations of *R. sanguineus* from China and USA. These findings suggest that these two populations are likely separated species, which supports the proposal that *R. sanguineus* tick complex may represent a species complex of at least two closely related species [24].

Nevertheless, another phylogenic study using 3 molecular markers: (16S, 12S and COX) detected 22 haplotypes, forming 4 different phylogenic groups and demonstrating that *R. sanguineus sensu lato* is related to the northern linage and *R. sanguineus* T2 is related to the southern linage. In addition to this two previous known lineages, another phylogenic clusters where formed, namely one constituted by the sequences isolated in *R. turanicus* from Italy Israel, and Switzerland and three others composed by *R. sanguineus* T1, *R. sanguineus* T3 and *R.sanguineus* T4. However, *R. sanguineus* T1 group was the one that assumed more relevance in terms of p-distance and in the number of the sequence that formed it, (mainly isolated from Greece). The distance within these four groups presented values of intra-specific variation, around 2,2% with the marker 16S, 2,8% with the marker 12S and 3,5% with the marker COX. However the values of inter-specific variation ranged from 3,3% to 18,1%, 3,5% to 15,3% and, 9,4% to 18,7%, in relation to 16S, 12S, and COX respectively. These values can be considered as a confirmation of the existence of at least 4 different groups, once these values are higher than the difference established between *R. sanguineus* and *R. guilhoni*. It is also noteworthy that phylogeny revealed the presence of different species under the name *R. turanicus*, once several of those are closer to *R. sanguineus*, and other were included within the southern linage [32].

At this point, it is possible to establish a comparison between the results acquired in this study and the ones previously obtained. The p-distance between the haplotypes obtained with the molecular marker 12S ranged between 0,28% and 0,83%, whereas and the p-distances, associated with the molecular marker 16S ranged, from 0,43% to 1,71% (excluding the haplotype related to control group). Both values indicate intra-specific variation; however the values associated with the 16S molecular marker pointed out to a higher level of intra-specific variation, although not enough to justify the classification in different species. This low value of intra-specific variability may be justified by the fact that the Portuguese populations of *R. sanguineus sensu lato* and *R. turanicus* present phenotypic differences among themselves, but not molecular differences [94]. Another possible explanation is related to the small sample considerated, for this study, in addition the sampling areas are associated with a more intense presence of *R. sanguineus* than *R. turanicus* [94, 104], which may contributed to a

underestimation of the genetic variability evidenced by the Portuguese populations of *R. sanguineus.*

Taking into consideration the existence of at least 4 different phylogenic groups: *R. sanguineus sensu lato* or Northern linage associated with tropical countries, *R. sanguineus* Type 1 associated mostly with Greece, *R. sanguineus* Type 2 or southern linage associated with temperate countries and *R. turanicus* [32]. The haplotypes obtained in this study relate to these groups as follows: the haplotypes obtained with 12S present p-distance that range from 9,15% to 9,76% when compared with Northern linage; 6,71% to 8,53% when compared to *R. turanicus* group; 7,01% to 7,93%, when compared to the group *R. sanguineus* T1 and finally from 0,00% to 1,52%, when compared with the to the group *R. sanguineus* T2. Similar results are acquired comparing with the haplotypes acquired with 16S, that present p-distances that range from; 7,66% to 10,21% when compared with the Northern linage; 8,51% to 10,6%, to the *R. turanicus* group; 4,68% to 7,66%, to the group *R. sanguineus* T1; from 0,43% to 2,55%, to the group *R. sanguineus* T2. Taking these values into consideration, as well as the phylogenic structure of the trees, (data displayed by the tables 11 and 12, and figs 34 and 35) it is possible to concluded that all haplotypes found in this study are inserted in the *R. sanguineus* T2 group and are genetic and phylogenic different from any of the other 3 phylogenic groups.

These results are coherent with the studies conducted earlier, for instance [32], when comparing the p-distance among the *R. sanguineus* T2 group with the other 3 groups, using the marker 16S, the following values, 8,7%, 12,4 % and 7% in relation to the northern linage, the *R. turanicus* groups and the *R. sanguineus* T1, respectively, were obtained. When using the marker 12S the values , 10,5%, 10,2 % and 10,4% in relation to the northern linage, the *R. turanicus* groups and the *R. sanguineus* T1, respectively, were acquired and when using the marker COX the values, 15,7%, 14,2 % and 12,5% in relation to the northern linage, the *R. turanicus* groups and the *R. sanguineus* T1, respectively, were obtained. Despite the fact of the distances being more marked in this study, particularly when the cox molecular marker is used the results support the same conclusions.

By focusing on the distance between northern and Southern linage, therefore the distance among sequences isolated in tropical countries and the ones isolated in areas with temperate climate, our values range from 7,66% to 10,21%, using 16S and between 9,15% and 9,76%

using 12S. Nava 2012 [88] presented values that ranged from 4,9% to 6,5%, using 16S and between, 7,6% to 8,5% using the 12S. Moraes-Filho 2011 [87], using the marker 16S, registered p-distances between these two clades that ranged from 5,85% to 6,96. One year earlier, Burlini 2010 [86] by comparing Brazilian sequences with European sequences obtained distances that reached 15,9% using 12S and 9,8% using 16S. Szabo 2005 [85] also obtained values that reached 8,3% p-distance when comparing sequences isolated in tropical areas with sequences isolated in temperate areas and even Beati and Kierings 2001 [18] registered differences of 7,7% when comparing French sequences (Southern linage) with African sequences (Northern linage). Despite some variation regarding the values, once in some studies the divergence is more marked than other, all these results obtained in several years and studies suggest that there are considerable genetic differences that separate the northern linage associated with tropical climate from the Southern linage related to temperate climate.

By analyzing the phylogenic trees created to infer the phylogeny of the genes 12S and 16S (fig 49 and 50 respectively) it is possible to see that in the tree associated to the 12S gene, the closest group to the group *R. sanguineus* T2, is *R. turanicus* group constituted by sequences isolated in Italy, Switzerland, and Israel, followed by the group *R. sanguineus* T1 formed with sequences isolated in Greece, and the phylogenetically more distant group, is the one constituted by sequences isolated in tropical countries, also known as *R. sanguineus sensu lato* group or northern linage. In turn, the tree associated to the 16S gene presents a similar structure but with several differences namely, the closest group to the *R. sanguineus* T2 group is the *R. sanguineus* T1 formed with sequences isolated in Greece and the farthest group, apart from the haplotypes added to this study as a control-group, is the *R. turanicus* group, constituted with sequences isolated in Italy. The fact that the tree inferred with the molecular marker 12S presents the exact same structure that the one presented in [32] supporting the information displayed on that tree. In turn when comparing, the tree inferred with the marker 16S with the one presented in that article, it is possible to note that the main difference refers to the fact the one presented in this study shows the *R. turanicus* as the farthest group from the southern linage, instead of the *R. sanguineus sensu lato* group, but that constituted the branch with the lowest bootstrap value, which may justified that difference, despite that the rest of the tree presents the same structure what also supports the information displayed in the tree inferred with 16S.

Both phylogenetic trees present the northern lineage associated with areas of the globe characterized by tropical climatic conditions, well separated from the Southern linage, related to areas of the globe characterized by temperate climatic conditions a result that supports previous findings [87, 88].

The differences observed between the phylogenic trees, are also something, that had already been described before and one possible explanation are the differences of size between the fragments of both genes and also, that 16S sequences are scarse in the GenBank than 12S sequences, what may induce some differences in the obtained results for both genes [86].

However, the differences between the two markers are not restricted to the structure of the trees, once they evidenced different performances. The marker 16S showed more discrimination, as it allowed to obtain more haplotypes than the marker 12S and especially because it was able to identify the specimens that were added to the study as control group, *R. pusillus*. By the opposite the marker 12S failed to identify those specimens as *R. pusillus.* It's possible that the better performance produced by the molecular marker 16S may result of the genetic sequence related to the marker 16S, being associate with more variation in the Portuguese's population of *R. sanguineus,* than the sequence connected to 12S marker.

Phylogenetic analysis also showed that some sequences morphologically classified as *R. turanicus* appear to be closely related to *R. sanguineus* s. l, an occurence previously noted [10, 32, 86]. What may suggest the existence of more than one species of *R. turanicus*, or that these sequences in the *R. turanicus* group, where actually extracted from *R. sanguineus,* misclassified from the start.

It is also noteworthy that the haplotypes 6, 1, 4 and 8 acquired with the 16S marker, in spite of being associated with p-distance values that do not justify the classification as a different species, are grouped in a separate tree branch, forming a kind of mini-clade. This isolation probably suggest the beginning of a speciation process, in a phase where there is not enough genetic variability to support the differentiation of the species, at the present time, but in the near future, that might occur, since there is already enough intra-variability to group some haplotypes in a separated branch.

One aspect that supports this hypothesis is the fact the different obtained haplotypes in this study, at the moment of the morphologic classification were classified as different morphologic groups, namely *R. turanicus, R. pusillus*, *R. sanguineus* T1, *R. sanguineus* T2, *R. sanguineus* s. s., and *R. sanguineus* Intermediate. So despite the fact that the genetic differences are not very pronounced yet, the morphologic differences, already justify different classifications, and probably that in the future the genetic differences will increase. Another situation that defends this theory is associated with the fact *R. sanguineus* and *R. turanicus* present distinct host preferences , once the Portuguese populations of *R. sanguineus* used in this study were found on dogs and *R. turanicus* are mostly found in ruminant cattle namely sheep [93], which may correspond to one of the first steps of speciation.

The understanding of such echoes is of great importance to public heath considering that the spectrum of tick borne diseases affecting domestic animals and humans has increased in recent years; many important zoonotic tick borne diseases, such as anaplasmosis, babesiosis, ehrlichiosis and Lyme borreliosis are increasingly gaining more attention from physicians and veterinarians. With the development of molecular biology, new species, strains or genetic variants of microorganisms are being detected in ticks worldwide, and the list of potential tick borne pathogens continues to increase [58].

Adicionally ticks are associated with hundreds of thousands of cases of illness in humans that are reported each year and it is believed that these numbers are underestimated, as consequence ticks are considered the second most important vector of human disease after mosquitoes [56].

To worsen this scenario it is believed that the risks of human exposure to ticks will continue to increase, as a result of the habitats fragmentation a due to human activity such as deforestation [69]. It is also believed that climate is playing a decisive role in the spread, seasonality and abundance of several ticks species with negative economic impact and in human and animal health climate changes associated with global warming are set to create new opportunities for the expansion of tick populations and to increase the numbers associated with human parasitism and illness [8, 105].

Particulary, Portugal displays ecological condition such as adequate vegetation and variety of suitable hosts and also favorable climatic conditions that benefit distribution and maintenance

of ticks and tick borne diseases [90]. Each year over 1000 cases of Boutounnese fever are registered representing one of the highest rates of incidence in Europe [91, 92].

So considering this prediction associated with climatic changes it's expected that in Portugal ticks populations will raise and its activity will increase and remain for longer periods of time, probably new species will colonize our country, and the number of cases of parasitism and disease in Portugal will increase severely. For such reasons it is very relevant to understand the Portuguese populations of *R. sanguineus* it's morphological and genetic variability and also the presence of *R. turanicus* in Portugal and how this two species are associated with different pathogens.

6. Conclusions and Future Perspectives

- This results support the idea that the spiracular plates in males and the genital aperture in females are indeed the more adequate structures to differentiate *R. sanguineus* from *R. Turanicus.*

- The study also holds that in males *R. turanicus* presents wider spiracles, and the ending spiracle tail is larger than half of the width of the adjacent festoon, in turn *R. sanguineus* presents thinner spiracles and the the ending spiracle tail is equal or less than half of the adjacent festoon. In females *R. sanguineus* shows a genital opening with the shape of an open U, with sclerites far apart from each other, on the other hand, *R. turanicus* exhibits a genital opening in the shaped of a close U, with sclerites closer to each other.

- Results demonstrated that there is a lot of morphological variability in the Portuguese populations of *R. sanguineus* once that the morphological analyses reveled the formation of 8 morphologic clusters in males, namely: *R. sanguineus sensu lato*, *R. sanguineus* T1, *R. sanguineus* T2, *R. turanicus*, and *R. pusillus* also the 3 groups not previously described (*R. sanguineus* D, *R. sanguineus* R. R. *turanicus* D), and the presence of 5 morphologic cluster in females, namely *R. sanguineus sensu lato*, *R. sanguineus* T1, *R. sanguineus* T2, *R. turanicus*, *R. pusillus* and one not previously described (*R. sanguineus* Intermediate). These morphologic clusters differ from each other in terms of several morphological structures, namely the spiracular plate and the genital region in females.

- It was also observable that the quantitative variables had stronger performance than the qualitative variables; however this difference was not so evident in females.

- The p-distance values obtained with the molecular markers 12S and 16S, ranged between 0,28% and 1,71%, indicating intra-specific variation; however the values associated with the 16S molecular marker point to a higher level of intra-specific variation, still not enough to justify the classification as different species.

- It is possible to concluded, that all haplotypes found in this study are inserted in the *R. sanguineus* T2 group and are genetic and phylogenic different from any of the other 3 phylogenic groups (*R. sanguineus* T1, *R. sanguineus sensu lato* and *R. turanicus*).

- Our results support the hypothesis, presented previously in several studies, that there are considerable genetic differences that separate the northern linage associated with tropical climate, from the Southern linage associated with temperate climate. Despite the fact that both lineages are considerated as *R. sanguineus*, the p-distance values above 7,8% indicated that we are definitely in the presence of two different species.

- The marker 16S showed more discrimination, once it allowed to obtain more haplotypes than the marker 12S and especially because it was able to identify the specimens that were added to the study as control group, *R. pusillus*. In turn, the marker 12S failed to identify those specimens as *R. pusillus.*

- It is also noteworthy that the haplotypes 6, 1, 4 e 8, acquired with the 16S marker, in spite of the fact that aren't associated with p-distance values that justify the classification as a different species, when a phylogenetic analysis is performed, it is possible to state that these haplotypes are grouped in a separate tree branch, forming a kind of mini-clade. Suggesting that it is possible that what it's being observed is the beginning of a speciation process, as such, there is not enough genetic variability, to support the distinction as different species, at the present time, but in the future, that might occur, once, there is already enough intra-variability that leads some haplotypes to group in a separated brunch.

- Future studies should focus on understanding the full extent that climatic change may cause in the Portuguese tick populations and also how it would affect the tick borne diseases incidence. Simultaneously, it would be interesting to conduct a study similar to this one but using *R. sanguineus* collected in dogs and *R. sanguineus* collected in others domestics animals namely cattle, and see if genetic difference would be higher, once it is described that *R. turanicus* prefer that type of host.

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Appendices

Males Qualitative Variables Clusters Characterization:

Cluster 1 – In the 83 elements (34.7% of the 239 male specimens) within this cluster**:** 68 (81,9%) present dense conscutum punctation distribution, 15 (18.1%) present sparse punctation distribution; 3 (3,6%) present small conscutum sized punctations, 62 (74,7%) have small and medium sized punctations, 18 (21.7%) have small, medium and large sized punctations; 81 (97,6%) present cervical fields depressions, 2 (2,4%) don't present apparent fields depression; 1 (1,2%) present a small cervical fields shape, 71 (85.5%) present a large and curved cervical fields shape, 11 (13.3%) present a large and straight cervical fields shape; 2 (2.4%) present small setiferous punctations, 81 (97.6%) present large setiferous punctations; 7 (8.4%) present mild cervical grooves, 76 (91.6%) present defined cervical grooves; 10 (12%) present short ventral palps, 69 (83,1%) present medium ventral palps, 4, (4,8%) present long ventral palps; 12 (14.5%) have the second palp square-shaped, 71 (85.5%) have the second palp long in width; 44 (53%) present lateral grooves beginning immediately after the eye, 39 (47%) present lateral grooves beginning distant of the eye; 2 (2,4%) have the lateral grooves ending before 1st festoon, 41 (49,4 %) have the lateral grooves ending in the 1st festoon, 40 (48.2 %) have the lateral grooves ending in the 2nd festoon; 27 (32.5%) present lateral grooves with punctuate texture, 56 (67,5%) present lateral grooves with distinctly punctate-texture; 6 (7,2%) present short posteromedian grooves, 77 (92,8%) present long posteromedian grooves; 11 (13,3%) present shallow posteromedian grooves, 72 (86,7%) present deep posteromedian grooves; 8 (9,6%) present shallow paramedian grooves, 75 (90,4%) present deep paramedian grooves; 56 (67,5%) present circular-shaped paramedian grooves, 21 (25,3%) present oval-shaped paramedian grooves, 6 (7,2%) present comma-shaped paramedian grooves; All members don't present parma; 47 (56,6%) present type 1 spiracular areas, 23 (27,1%) present type 2 spiracular area, 13 (15,7%) present type 3 spiracular areas; 26 (31,3%) present adanal plates posterior margin square-shaped, 57 (68,7%) present adanal plates posterior margin round-shaped; 5 (6%) present adanal plates total form square-shaped, 7 (8,4%) present adanal plates total form round-shaped, 71 (85,5%) present adanal plates total form with intermediate form between round and square-shape; 10 (12%) present a short adanal plates end, 73 (88%) present a long adanal plates end.

Cluster 2 – In the 122 elements (51% of the 239 male specimens) within this cluster**:** 96 (78,7%) present dense conscutum punctation distribution, 26 (21,3%) present sparse punctation distribution; 6 (4,9%) present small conscutum sized punctations, 92 (75,4%) have small and medium sized punctations, 24 (19,7%) have small, medium and large sized punctations; 122 (99,2%) present cervical fields depressions, 1 (0,8%) don´t present apparent fields depression; 1 (0,8%) present a small cervical fields shape, 102 (83,6%) present a large and curved cervical fields shape, 19 (15,6%) present a large and straight cervical fields shape; 3 (2,5%) present small setiferous punctations, 119 (97,5%) present large setiferous punctations; 7 (5,7%) present mild cervical grooves, 115 (94,3%) present defined cervical grooves; 14 (11,5%) present short ventral palps, 104 (85,2%) present medium ventral palps, 7 (5,7%) present long ventral palps; 13 (10,7%) have the second palp square-shaped, 104 (85,2%) have the second palp long in width, 5 (4,1%) have the second palp long in length; 61 (50%) present lateral grooves beginning immediately after the eye, 61 (50%) present lateral grooves beginning distant of the eye; 2 (1,6%) have the lateral grooves ending before 1st festoon, 62 (50,8 %) have the lateral grooves ending in the 1st festoon, 58 (47,5 %) have the lateral grooves ending in the 2nd festoon; 34 (27,9%) present lateral grooves with punctuate texture, 88 (72,1%) present lateral grooves with distinctly punctate-texture; 12 (9,8%) present short posteromedian grooves, 110 (90,2%) present long posteromedian grooves; 13 (10,7%) present shallow posteromedian grooves, 109 (89,3%) present deep posteromedian grooves; 10 (8,2%) present shallow paramedian grooves, 112 (91,8%) present deep paramedian grooves; 91 (74,6%) present circular-shaped paramedian grooves, 28 (23%) present oval-shaped paramedian grooves, 3 (2,5%) present comma-shaped paramedian grooves; All members present parma; 91 (73,8%) present type 1 spiracular areas, 28 (23%) present type 2 spiracular area, 4 (3,3) present type 3 spiracular areas; 43 (35,1%) present adanal plates posterior margin square-shaped, 79 (74,8%) present adanal plates posterior margin round-shaped; 19 (15,6%) present adanal plates total form square-shaped, 11 (9%) present adanal plates total form round-shaped, 92 (75,4%) present adanal plates total form with intermediate form between round and square-shape; 19 (15,6%) present a short adanal plates end, 103 (84,4%) present a long adanal plates end.

Cluster 3 – In the 34 elements (14,2% of the 239 male specimens) within this cluster**:** 33 (97,1%) present dense conscutum punctation distribution, 1 (2,9%) present sparse punctation distribution; 9 (26,5%) present small conscutum sized punctations, 21 (61,8%) have small and medium sized punctations, 4 (11,8%) have small, medium and large sized punctations; 27

(79,4%) present cervical fields depressions, 7 (20,6%) don't present apparent fields depression; 6 (17,6%) present a small cervical fields shape, 22 (64,7%) present a large and curved cervical fields shape, 6 (17,6%) present a large and straight cervical fields shape; 6 (17,6%) present small setiferous punctations, 3 (8,8%) present large setiferous punctuations, 25 (73,5%) present small and large setiferous punctations; 8 (23,5%) present mild cervical grooves, 26 (76,5%) present defined cervical grooves; 20 (58,8%) present short ventral palps, 11 (32,4%) present medium ventral palps, 3 (8,8%) present long ventral palps; 5 (14,7%) have the second palp square-shaped, 28 (82,4%) have the second palp long in width, 1 (2,9%) have the second palp long in length; 19 (55,9%) present lateral grooves beginning immediately after the eye, 15 (44,1%) present lateral grooves beginning distant of the eye; 18 (52,9 %) have the lateral grooves ending in the 1st festoon, 15 (44,1%) have the lateral grooves ending in the 2nd festoon; 1 (2,9%) present lateral grooves with smooth texture, 14 (41,2%) present lateral grooves with punctuate texture, 19 (55,9%) present lateral grooves with distinctly punctate-texture; 16 (47,1%) present short posteromedian grooves, 18 (52,9%) present long posteromedian grooves; 6 (17,6%) present shallow posteromedian grooves, 28 (82,4%) present deep posteromedian grooves; 9 (26,5%) present shallow paramedian grooves, 25 (73,5%) present deep paramedian grooves; 25 (73,5%) present circular-shaped paramedian grooves, 7 (20,6%) present oval-shaped paramedian grooves, 2 (5,9%) present comma-shaped paramedian grooves; 18 (52,9%) off the members present parma, 16 (47,1%) don´t present parma; 15 (44,1%) present type 1 spiracular areas, 11 (32,4%) present type 2 spiracular area, 8 (23,5) present type 3 spiracular areas; 9 (26,5%) present adanal plates posterior margin square-shaped, 25 (73,5%) present adanal plates posterior margin round-shaped; 2 (5,9%) present adanal plates total form square-shaped, 4 (11,8%) present adanal plates total form round-shaped, 28 (82,4%) present adanal plates total form with intermediate form between round and square-shape; 22 (64,7%) present a short adanal plates end, 12 (35,3%) present a long adanal plates end.

Females Qualitative Variables Clusters Characterization:

Cluster 1 – In the 49 elements (20,8% of the 236 female specimens) within this cluster: 1 2,0% have sparse scutum punctuation distribution, 48 (98,0%) have dense scutum punctuation distribution; 12 (24,5%) have small scutum punctuation size 32 (65,3%) have medium scutum punctuation size, 5 (10,2%) have large scutum punctuation size; 18 (36,7%) have slightly sinuous scutum posterior margin shape, 31 (63,3%) present sinuous scutum posterior margin; 46 (93,9%) present apparent cervical fields depression, 3 (6,1%) of the specimens do not present apparent cervical fields depression; 8 (16,3%) have small cervical fields, 35 (71,4) have large and curved cervical fields shape, 6 (12,2%) have large and straight cervical fields shape; 6,1% (3) have small setiferous punctuations on the cervical fields, 7 (14,3%) have large setiferous punctuations on the cervical fields, 39 (79,6%) present small and large setiferous punctuations on the cervical fields, 42 (85,7%) present defined cervical grooves, 7 (14,3%) have mild cervical grooves; 7 (14,3%) have a square shaped second palp, 40 (81,6%) have second palp long in width, 2 (4,1%) have second palps long in height, 21 (42,9%) display the pattern 1 as the form of genital aperture, 28 (57,1%) display the pattern 2 as the form of the genital aperture

Cluster 2 – In the 154 elements (64,4% of the 236 female specimens) within this cluster: 32 (21,1%) have sparse scutum punctuation distribution, 119 (78,3%) have dense scutum punctuation distribution, 1 (0,7%) have localized dense scutum punctuation; 5 (3,3%) have small scutum punctuation size, 122 (80,3%) have medium scutum punctuation size, 25 (16,4%) have large scutum punctuation size; 68 (44,7%) have slightly sinuous scutum posterior margin shape, 84 (55,3%) present sinuous scutum posterior margin; 149 (98%) present apparent cervical fields depression, 2 (3%) of the specimens present mild cervical fields depression; 2 (1,3%) present small cervical fields shape, 118 (77,6%) have large and curved cervical fields shape; 32 (21,1) have large and straight cervical fields shape; 2 (1,3%) large setiferous punctuations on the cervical fields, 150 (98,7%) present large and small setiferous punctuations on the cervical fields, 141 (92,8%) present defined cervical grooves, 4 (2,6%) present mild cervical grooves; 29 (19,1%) have square shaped second palps, 119 (78,3) have second palps long in width, 4 (2,6%) have second palps long in height; 22 (14,5%) display the pattern 2 as the form of genital aperture, 105 (69,1%) display the

pattern 3 as the form of the genital aperture, 16 (10,5%) display the pattern 4 as the form of genital aperture, 9 (5,9%) display the pattern 5 as the form of the genital aperture.

Cluster 3 – In the 35 elements (14,8% of the 236 female specimens) within this cluster: 2 (5,7%) have sparse scutum punctuation distribution, 33 (93,4%) have dense scutum punctuation distribution; 2 (5,7%) have small scutum punctuation size, 30 (85,7%) have medium scutum punctuation size, 3 (8,6%) have large scutum punctuation size; 14 (40,0%) have slightly sinuous scutum posterior margin shape, 21 (60,0%) present sinuous scutum posterior margin; 34 (97,1%) present defined cervical fields depression, 1 (2,9%) of the specimens present mild fields depression; 27 (77,1%) have large and curved cervical fields shape; 8 (22,9%) have large and straight cervical fields shape; 34 (97,1%) have small and large setiferous punctuations on the cervical fields, 1 (2%) present large setiferous punctuations on the cervical fields; 30 (85,7%) present defined cervical grooves, 5 (14,3%) present mild cervical grooves; 6 (17,1%) have square shaped second palps, 27 (77,1%) have second palps long in width, 2 (5,7%) have second palps long in height; 2 (5,7%) display the pattern 1 as the form of genital aperture, 14 (40,0%) display the pattern 2 as the form of genital aperture, 13 (37,1%) display the pattern 3 as the form of the genital aperture, 13 (37,1%) display the pattern 4 as the form of genital aperture, 3 (8,6%) display the pattern 5 as the form of the genital aperture.

Table 4 – Information of each male element of the sample: relative to the taxonomic group to which it belongs as well as the qualitative and quantitative clusters, where it was previously inserted, alongside with the region where it was collected and the identifying numbers of each specimen.

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1594	Sl	1	1	T2	1971	VFX	2	1	T1
1596	Sl	1	1	T2	1972	VFX	1	2	Af
1597	Sl	2	2	T1	1973	VFX	1	1	T2
1598	Sl	2	1	T1	1974	VFX	2	1	T2
1599	Sl	1	2	T1	1975	VFX	1	1	Af
1603	Sl	2	2	T2	1976A	VFX	2	1	T2
1611	Sl	3	1	Tur	1976B	VFX	2	1	T2
1612	Sl	1	2	Af	1979	VFX	2	1	T1
1613	Sl	1	1	Af	1984	VFX	2	2	Af
1614	Sl	1	1	Af	1985	VFX	1	2	T1
1618	Sl	1	1	D	1986	VFX	1	1	T1
1621	Sl	2	2	T2	1987	VFX	3	1	Tur
1622	Sl	2	2	T2	1992	VFX	3	1	T1
1624	Sl	1	2	T2	1993	VFX	2	1	T1
1626	Sl	2	2	R	1994	VFX	2	2	T2
1627	Sl	2	2	Af	1995	VFX	2	2	T2
1628	Sl	1	3	Af	1996	VFX	3	1	Tur D
1629	Sl	3	1	T2	1997	VFX	2	2	T2
1630	Sl	3	3	T1	2000	VFX	2	2	T1
1631	Sl	2	1	T2	2005	VFX	2	1	T2
1632	Sl	2	2	R	2007	VFX	2	2	T1
1637	Sl	1	3	T1	2010	VFX	2	3	T2
1641	Sl	1	2	Af	2011	VFX	2	1	T2
1643	Sl	2	3	T2	2012	VFX	2	2	T1
1646	Sl	2	1	T1	2014	VFX	1	1	Af
1650	Sl	2	2	T1	2017	VFX	2	1	T1

Table 4 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1652	Sl	1	1	T1	2018	VFX	1	2	T1
1654	Sl	2	1	R	2021	VFX	1	1	T1
1658	Sl	1	2	T1	2025	VFX	2	1	T1
1659	Sl	2	2	T2	2029	VFX	3	3	T1
1661	Sl	1	2	Af	2034	VFX	1	2	Af
1662	Sl	1	2	T1	2036	VFX	3	2	T2
1671	Sl	1	2	T1	2037	VFX	1	2	Af
1672	Sl	1	2	D	2061	VFX	2	1	T2
1673	Sl	1	2	T1	2063	VFX	2	2	T2
1675	Sl	1	2	T1	2064	VFX	3	1	Tur
1676	Sl	2	2	T1	2065	VFX	3	2	T2
1677	Sl	2	2	T1	2066	VFX	2	2	T2
1680	Sl	1	2	Af	2067	VFX	1	2	Af
1682	Sl	1	2	T1	2077	VFX	1	2	Af
1684	Sl	1	2	T2	2078	VFX	1	3	D
1685	Sl	3	2	Tur	2083	VFX	1	1	T1
1687	Sl	2	2	T1	2084	VFX	1	2	D
1690	Sl	2	2	T2	2085	VFX	1	2	Af
1691	Sl	2	2	T1	2086	VFX	3	2	T2
1692	Sl	3	2	T2	2087	VFX	1	2	Af
1693	Sl	1	1	Af	2094	VFX	1	2	T1
1694	Sl	2	2	T2	2095	VFX	1	2	D
1695	Sl	2	2	T1	2096	VFX	2	2	T2
1696	Sl	1	3	T1	2097	VFX	2	3	T1
1697	Sl	2	2	T1	2100	VFX	1	2	T1
1698	Sl	1	3	T1	2102	VFX	2	2	T2
1701	Sl	1	2	Af	2103	VFX	3	2	Tur
1704	Sl	1	3	Af	2110	VFX	1	2	T1

Table 4 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1705	Sl	3	1	Tur	2111	VFX	3	3	Tur D
1719	Sl	3	2	Tur	2116	VFX	2	2	T1
1720	Sl	2	1	T1	2117	VFX	2	2	T2
1722	Sl	1	3	T2	2118	VFX	2	2	D
1727	Sl	2	2	T1	2119	VFX	1	2	D
1733	Sl	1	2	Af	2120	VFX	1	2	T1
1735	Sl	1	3	T2	2121	VFX	2	2	T2
1736	Sl	1	1	T2	2122	VFX	1	2	D
1738	Sl	1	1	T1	2123	VFX	1	2	Af
1739	Sl	1	3	T1	2124	VFX	1	2	T1
1740	Sl	1	3	Af	2125	VFX	2	2	T1
1741	Sl	2	1	T1	2129	VFX	1	1	T2
1742	Sl	2	1	T1	2130	VFX	2	1	R
1743	Sl	1	1	T2	2136	VFX	2	1	T2
1747	Sl	1	1	Af	2138	VFX	2	1	T1
1751	Sl	2	2	T1	2140	VFX	2	1	T2
1754	Al	3	3	Pus	2144	VFX	2	2	Tur
1755	Al	3	3	Pus	2143	VFX	1	2	T1
1764	Al	2	2	D	2154	VFX	2	2	T1
1765	Al	3	2	Tur	2155	VFX	2	1	T2
1766	Al	3	3	Tur	2159	VFX	3	1	Tur D
1768	Al	1	1	Af	2160	VFX	2	2	T2
1769	Al	3	2	Tur	2163	VFX	2	2	T2
1770	Al	1	1	Af	2175	VFX	1	2	T1
1806	P	2	2	T2	2176	VFX	1	2	T1
1807	P	1	2	T1	2177	VFX	2	1	T1
1808	P	2	2	T2	2182	VFX	3	1	Pus
1809	P	2	2	T2	2183	VFX	1	1	T1

Table 4 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1810	P	2	2	T2	2184	VFX	1	1	Af
1811	P	3	3	Pus	2197	VFX	2	2	T1
1813	ALC	3	3	Tur	2198	VFX	1	2	T2
1814	ALC	2	2	Tur	2226	VFX	2	2	T2
1815	ALC	3	3	Pus	2227	VFX	2	1	T2
1820	ALC	3	1	Tur	2232	VFX	3	1	Tur
1821	ALC	3	1	Tur	2233	VFX	1	2	D
1830	ALC	2	3	T1	2236	VFX	3	2	T2
1831	ALC	2	1	T1	2242	VFX	3	2	Tur
1832	ALC	2	2	T1	2244	VFX	3	2	Tur
1834	ALC	3	3	Tur	2272	VFX	2	2	T2
1837	ALC	3	1	Tur	2305	VFX	2	1	T1
1838	ALC	3	1	Tur	2306	VFX	1	1	T2
1840	ALC	3	1	Tur	2307	VFX	2	2	T2
1842	ALC	2	1	T1	2308	VFX	1	3	T2
1844	ALC	1	3	T1	2309	VFX	2	2	T2
1847	ALC	1	1	T2	2310	VFX	1	1	T2
1864	ALC	3	2	Tur	2313	VFX	2	1	T2
1865	ALC	3	1	T2	2330	VFX	1	2	T1
1866	ALC	3	3	Pus	2337	VFX	2	1	T2
1867	ALC	3	1	Pus	2372	VFX	1	1	T2
1868	ALC	3	3	Pus	2373	VFX	2	1	T2
1869	ALC	3	3	Pus	2376	VFX	2	2	T1
1870	ALC	3	3	Pus	2377	VFX	2	2	T1
1875	ALC	1	2	T2	2378	VFX	1	2	T1
1876	ALC	3	2	T2	2379	VFX	2	2	T1
1877	ALC	1	2	T2	2380	VFX	2	1	T1
1882	ALC	3	2	Tur	2385	VFX	3	2	Tur

Table 4 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1887	ALC	2	3	T2	2388	VFX	3	1	Tur
1888	ALC	3	3	T2	2389	VFX	3	1	Tur
1899	ALC	2	2	T2	2401	VFX	2	2	T2
1903	ALC	2	2	T2	2402	VFX	2	2	R
1907	ALC	2	1	T1	2403	VFX	2	1	T1
1957	ALC	3	1	T2	2404	VFX	1	1	T1
1959	ALC	1	2	D	2405	VFX	2	3	T1
1969	VFX	2	1	T1	2406	VFX	2	2	T2
1970	VFX	3	2	T1	2407	VFX	2	3	D
					2408	VFX	2	1	T2

Note: INS- Identification Number of the Specimen, **R-** Region where the specimen was collected, **QTL-** Quantitative Cluster, **QLC-** Qualitative Cluster, **MC-** Morphologic Cluster, Sl- Setubal, Al- Alcochete, P- Peniche, ALC, Alcobaça, VFX- Vila Franca de Xira, Af- *R. Sanguineus Sensum Lactum*, T1- *R. Sanguineus* Type 1, T2- *R. Sanguineus* Type 2, R- *R. Sanguineus* R, D- *R. Sanguineus* D, Pus- *R. Pusillus*, Tur- *R. Turanicus*, Tur D- *R. Turanicus* D.

Table 8 – Information of each female element of the sample. Relative to the taxonomic group to which it belongs as well as the qualitative and quantitative clusters, where it was previously inserted, alongside with the region where it was collected and the identifying numbers of each specimen.

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1595	SL	1	1	Af	1955	ALC	1	2	T2
1604	SL	2	1	Pus	1956	ALC	1	2	T2
1605	SL	1	1	Af	1958	ALC	3	1	T1
1608	SL	1	2	Int	1960	ALC	1	2	Int
1610	SL	2	2	Tur	1961	ALC	1	2	T2
1615	SL	1	2	T2	1977	VFX	1	2	T2
1633	SL	3	2	T2	1978	VFX	1	3	T2
1634	SL	2	2	Af	1980	VFX	3	2	T2
1635	SL	1	3	Af	1983	VFX	1	3	Af
1644	SL	2	2	T2	1990	VFX	2	1	T1
1645	SL	3	2	T2	1998	VFX	2	1	T1
1649	SL	1	2	T2	1999	VFX	1	2	T2
1651	SL	2	1	Af	2001	VFX	1	2	T2
1653	SL	1	3	Int	2002	VFX	2	3	T1
1655	SL	1	2	T2	2003	VFX	3	1	T1
1656	SL	1	2	T2	2006	VFX	1	2	T2
1657	SL	1	2	T2	2008	VFX	1	2	Int
1660	SL	1	2	T2	2009	VFX	1	3	T2
1664	SL	1	2	T2	2013	VFX	1	3	T2
1665	SL	2	2	T2	2015	VFX	1	2	T2
1667	SL	1	3	Af	2016	VFX	1	2	T2
1670	SL	3	2	T2	2019	VFX	1	2	T2
1674	SL	2	2	T2	2020	VFX	1	2	T2
1678	SL	1	2	T2	2022	VFX	1	3	T2
1679	SL	1	1	Af	2023	VFX	1	2	T2
1681	SL	1	2	T2	2024	VFX	1	1	Af
1683	SL	1	2	T2	2030	VFX	1	1	Af
1689	SL	1	2	T2	2031	VFX	1	2	T2
1699	SL	3	2	T2	2032	VFX	1	1	Af
1700	SL	1	2	Int	2033	VFX	1	2	T2
1702	SL	2	3	Int	2035	VFX	1	2	T2
1706	SL	1	2	T2	2038	VFX	1	2	T2
1708	SL	2	2	Af	2039	VFX	1	3	T2
1709	SL	1	2	T2	2040	VFX	3	2	T2
1710	SL	1	3	Int	2041	VFX	1	2	T2
1711	SL	1	2	T2	2042	VFX	1	2	T2
1712	SL	1	2	Int	2043	VFX	3	3	T1
1716	SL	1	2	Int	2045	VFX	1	1	Af
1718	SL	1	2	Int	2051	VFX	2	2	T2
1721	SL	1	2	T2	2055	VFX	1	2	Int

Table 8 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	MC
1724	SL	2	1	T1	2057	VFX	1	2	Af
1730	SL	1	3	T2	2058	VFX	2	2	Af
1731	SL	2	3	Tur	2060	VFX	1	2	Int
1732	SL	1	2	T2	2062	VFX	1	2	T2
1734	SL	1	2	T2	2075	VFX	1	1	Af
1737	SL	1	2	Int	2082	VFX	1	2	T2
1745	SL	3	2	T1	2092	VFX	3	1	Af
1745(2)	SL	1	3	T2	2098	VFX	2	2	T2
1746	SL	1	2	T2	2099	VFX	1	2	Af
1748	SL	1	2	T2	2104	VFX	1	1	Af
1749	SL	1	2	T2	2107	VFX	2	2	T2
1750	SL	1	2	T2	2108	VFX	2	3	Tur
1753	SL	3	2	T1	2114	VFX	1	2	Int
1756	AL	2	2	Tur	2126	VFX	1	2	T2
1757	AL	2	1	Pus	2127	VFX	1	2	T2
1758	AL	2	1	Pus	2128	VFX	1	2	T2
1759	AL	2	2	Int	2131	VFX	3	2	T2
1761	AL	3	1	T1	2133	VFX	1	2	T2
1771	AL	3	3	T1	2134	VFX	3	2	T2
1772	AL	3	2	T1	2135	VFX	3	1	T1
1773	AL	3	2	T1	2137	VFX	3	2	T2
1774	AL	1	2	T2	2139	VFX	1	2	T2
1775	AL	1	2	T2	2141	VFX	2	3	T1
1776	AL	2	2	Int	2145	VFX	1	2	T2
1778	AL	1	2	T2	2150	VFX	1	2	T2
1794	P	1	2	T2	2151	VFX	1	3	Af
1801	P	3	2	T2	2152	VFX	1	2	T2
1802	P	3	2	T1	2153	VFX	1	1	Pus
1803	P	3	1	T1	2157	VFX	2	2	Tur
1804	P	1	1	Af	2162	VFX	1	2	T2
1805	P	3	3	T2	2174	VFX	3	1	T1
1816	ALC	2	1	Pus	2178	VFX	2	1	Pus
1819	ALC	1	3	Af	2181	VFX	2	1	Pus
1824	ALC	1	2	T2	2186	VFX	1	1	Af
1829	ALC	2	2	Tur	2190	VFX	1	2	T2
1833	ALC	3	2	T2	2192	VFX	1	2	T2
1835	ALC	1	2	T2	2196	VFX	1	2	T2
1836	ALC	1	2	T2	2193	VFX	1	2	T2
1839	ALC	2	2	Tur	2195	VFX	1	2	Int
1841	ALC	2	2	Tur	2199	VFX	1	2	Af
1845	ALC	2	3	Tur	2202	VFX	2	2	Int
1846	ALC	2	2	Tur	2205	VFX	2	1	Pus
1848	ALC	1	2	T2	2208	VFX	2	1	Pus
1849	ALC	1	2	Af	2209	VFX	2	1	Pus

Table 8 – (Continued)

INS	R	QTC	QLC	MC	INS	R	QTC	QLC	
1850	ALC	2	3	T1	2210	VFX	2	3	Pus
1851	ALC	1	2	T2	2211	VFX	1	3	Pus
1852	ALC	1	2	T2	2212	VFX	2	1	Pus
1854	ALC	3	2	T1	2213	VFX	1	1	Pus
1855	ALC	1	3	Int	2214	VFX	1	1	Pus
1856	ALC	3	3	T2	2215	VFX	1	1	Pus
1858	ALC	1	2	T1	2216	VFX	1	1	Pus
1860	ALC	2	1	Pus	2219	VFX	1	2	T2
1861	ALC	2	1	Pus	2220	VFX	1	3	Af
1871	ALC	1	2	Af	2224	VFX	2	2	Af
1873	ALC	1	2	T2	2245	VFX	2	2	Af
1874	ALC	2	1	Pus	2246	VFX	1	1	Af
1878	ALC	3	2	T2	2248	VFX	1	2	T2
1879	ALC	1	2	T2	2249	VFX	3	1	T1
1880	ALC	1	2	T2	2257	VFX	3	2	T1
1881	ALC	2	3	T1	2258	VFX	1	2	Af
1883	ALC	1	2	Af	2303	VFX	2	1	Af
1884	ALC	1	2	T2	2304	VFX	1	2	T2
1885	ALC	1	2	T2	2311	VFX	3	1	Af
1890	ALC	1	3	T2	2318	VFX	1	2	T2
1891	ALC	3	1	T1	2340	VFX	1	2	T2
1892	ALC	1	2	T2	2343	VFX	3	1	Af
1893	ALC	3	1	Af	2349	VFX	1	2	Int
1894	ALC	1	2	T1	2352	VFX	1	2	T2
1896	ALC	1	2	T2	2364	VFX	1	2	T2
1897	ALC	1	2	T2	2367	VFX	1	2	T2
1901	ALC	1	3	T1	2387	VFX	2	2	Tur
1904	ALC	1	2	T2	2390	VFX	2	2	Tur
1906	ALC	1	3	T2	2391	VFX	1	2	Af
1909	ALC	1	2	T2	2392	VFX	1	1	Af
1951	ALC	1	3	Af	2396	VFX	3	2	T2
1952	ALC	1	2	T2	2397	VFX	2	2	T2
1953	ALC	3	1	T1	2398	VFX	1	2	T2
1954	ALC	2	3	T2	2399	VFX	1	2	Int

Note: INS- Identification Number of the Specimen, **R-** Region where the specimen was collected, **QTL-** Quantitative Cluster, **QLC-** Qualitative Cluster, **MC-** Morphologic Cluster, Sl- Setubal, Al- Alcochete, P- Peniche, ALC, Alcobaça, VFX- Vila Franca de Xira, Af- *R. Sanguineus*, T1- *R. Sanguineus* Type 1, T2- *R. Sanguineus* Type 2, Int- *R. Sanguineus* Intermediate

Matrix of absolute nucleotide differences and p-distance 12S

Table 16 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in italics, between all the sequences isolated by the 12S rDNA gene in this study.

	1594	2118	1838	1637	2130	1641	2140	1867	2380	1957	2405	1959	1693	1629	1992	1755	2159	2086	1807	2111	2385	1756	1841	1845	1846	1860	1861	1990	1999	2002	2045	2114	1609	1634	1700	1712	1774	1851	1891
1594		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2118	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1838	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1637	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2130	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1641	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2140	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1869	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2380	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1957	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2405	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1959	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1693	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**		*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,83 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,83 %*	*0,83 %*
1629	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1992	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1755	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2159	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2086	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1807	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2111	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,56 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,56 %*	*0,56 %*
2385	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1756	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*
1841	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*
1845	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**		*0,00 %*	*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*
1846	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**		*0,00 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*
1860	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**		*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,55 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*
1861	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**1**		*0,55 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,83 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,55 %*	*0,00 %*	*0,00 %*
1990	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**		*0,00 %*	*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*

Table 16 – (Continued)

	1594	2118	1838	1637	2130	1641	2140	1867	2380	1957	2405	1959	1693	1629	1992	1755	2159	2086	1807	2111	2385	1756	1841	1845	1846	1860	1861	1990	1999	2002	2045	2114	1609	1634	1700	1712	1774	1851	1891
1999	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**0**		*0,00 %*	*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2002	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**0**	**0**		*0,00 %*	*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2045	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**0**	**0**	**0**		*0,28 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
2114	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**3**	**1**	**1**	**1**	**1**		*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,28 %*	*0,83 %*	*0,83 %*
1609	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**0**	**0**	**0**	**0**	**1**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1634	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**0**	**0**	**0**	**0**	**1**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,55 %*	*0,55 %*
1700 1634	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**11**	**11**	**11**	**11**	**22**	**00**	**00**	**00**	**00**	**11**	**00**	**0**	*0,00 %*	*0,00 %0,00%*	*0,00 %0,00%*	*0,55 %0,55%*	*0,55 %0,55%*
1712 1700	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**11**	**11**	**11**	**11**	**22**	**00**	**00**	**00**	**00**	**11**	**00**	**00**	**0**	*0,00 %*	*0,00 %0,00%*	*0,55 %0,55%*	*0,55 %0,55%*
1774 1712	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**00**	**11**	**11**	**11**	**11**	**11**	**22**	**00**	**00**	**00**	**00**	**11**	**00**	**00**	**00**	**0**	*0,00 %*	*0,55 %0,55%*	*0,55 %0,55%*
1851 1774	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**31**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**11**	**11**	**11**	**11**	**11**	**02**	**20**	**20**	**20**	**20**	**31**	**20**	**20**	**20**	**20**	**2**	*0,55 %*	*0,00 %0,55%*
1891 1851	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**33**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**22**	**11**	**11**	**11**	**11**	**11**	**00**	**22**	**22**	**22**	**22**	**33**	**22**	**22**	**22**	**22**	**22**	**0**	*0,00 %*
1891	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**0**	**2**	**2**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**0**	

Note: P-distance= (number of different nucleotides/ Total number of analyzed nucleotides.

Matrix of absolute nucleotide differences and p-distance 16S

Table 17 – Matrix of absolute nucleotide differences (in bold) and matrix of p-distance in italics, between all the sequences isolated by the 16S rDNA gene in this study.

	1834	1838	1869	1671	1947	1959	2064	2086	2111	2118	2130	2380	1629	1755	2159	2242	2385	1610	1841	1845	1846	1860	1990	1999	2002	2045	2114	1609	1634	1700	1712	1774	1848	1851	1891
1834		*0,43 %*	*9,83 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,85 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1838	**1**		*10,26 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*1,28 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*9,87 %*	*0,00 %*	*0,43 %*	*0,43 %*	*9,87 %*	*0,00 %*	*0,43 %*	*0,00 %*	*10,26 %*	0,43 %	*0,43 %*	*0,85 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,85 %*	*0,85 %*	*1,28 %*
1869	**23**	**24**		*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*10,68 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*0,42 %*	*10,26 %*	*9,83 %*	*9,83 %*	*0,42 %*	*10,26 %*	*9,83 %*	*10,26 %*	*0,84 %*	9,83 %	*9,83 %*	*10,26 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*10,26 %*	*10,26 %*	*10,68 %*
1671	**0**	**1**	**23**		*0,00 %*	*0,00 %*	*0,00 %*	*0,85 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1947	**0**	**1**	**23**	**0**		*0,00 %*	*0,00 %*	*0,85 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1959	**0**	**1**	**23**	**0**	**0**		*0,00 %*	*0,85 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2064	**0**	**1**	**23**	**0**	**0**	**0**		*0,85 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2086	**2**	**3**	**25**	**2**	**2**	**2**	**2**		*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*10,30 %*	*1,28 %*	*0,85 %*	*0,85 %*	*10,30 %*	*1,28 %*	*1,71 %*	*1,28 %*	*10,68 %*	0,85 %	*0,85 %*	*1,28 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*1,28 %*	*1,28 %*	*1,71 %*
2111	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2118	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2130	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**		*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2380	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**		*0,00 %*	*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1629	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**		*9,44 %*	*0,43 %*	*0,00 %*	*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1755	**22**	**23**	**1**	**22**	**22**	**22**	**22**	**24**	**22**	**22**	**22**	**22**	**22**		*9,87 %*	*9,44 %*	*9,44 %*	*0,00 %*	*9,87 %*	*9,44 %*	*9,87 %*	*0,85 %*	9,44 %	*9,44 %*	*9,87 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,87 %*	*9,87 %*	*10,30 %*
2159	**1**	**0**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**		*0,43 %*	*0,43 %*	*9,87 %*	*0,00 %*	*0,43 %*	*0,00 %*	*10,26 %*	0,43 %	*0,43 %*	*0,85 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,85 %*	*0,85 %*	*1,28 %*
2242	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**		*0,00 %*	*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2385	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**		*9,44 %*	*0,43 %*	*0,85 %*	*0,43 %*	*9,83 %*	0,00 %	*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1610	**22**	**23**	**1**	**22**	**22**	**22**	**22**	**24**	**22**	**22**	**22**	**22**	**22**	**0**	**23**	**22**	**22**		*9,87 %*	*9,44 %*	*9,87 %*	*0,85 %*	9,44 %	*9,44 %*	*9,87 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,44 %*	*9,87 %*	*9,87 %*	*10,30 %*
1841	**1**	**0**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**	**0**	**1**	**1**	**23**		*0,43 %*	*0,00 %*	*10,26 %*	0,43 %	*0,43 %*	*0,85 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,85 %*	*0,85 %*	*1,28 %*
1845	**2**	**1**	**23**	**2**	**2**	**2**	**2**	**4**	**2**	**2**	**2**	**2**	**2**	**22**	**1**	**2**	**2**	**22**	**1**		*0,43 %*	*9,83 %*	0,85 %	*0,85 %*	*1,28 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*0,85 %*	*1,28 %*	*1,28 %*	*1,71 %*
1846	**1**	**0**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**	**0**	**1**	**1**	**23**	**0**	**1**		*10,26 %*	0,43 %	*0,43 %*	*0,85 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,85 %*	*0,85 %*	*1,28 %*
1860	**23**	**24**	**2**	**23**	**23**	**23**	**23**	**25**	**23**	**23**	**23**	**23**	**23**	**2**	**24**	**23**	**23**	**2**	**24**	**23**	**24**		9,83 %	*9,83 %*	*10,26 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*9,83 %*	*10,26 %*	*10,26 %*	*10,68 %*
1990	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**		*0,00 %*	*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1999	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**		*0,43 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2002	**1**	**2**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**	**2**	**1**	**1**	**23**	**2**	**3**	**2**	**24**	**1**	**1**		*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,43 %*	*0,85 %*	*0,85 %*	*1,28 %*
2045	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
2114	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*

Table 17 – (Continued)

	1834	1838	1869	1671	1947	1959	2064	2086	2111	2118	2130	2380	1629	1755	2159	2242	2385	1610	1841	1845	1846	1860	1990	1999	2002	2045	2114	1609	1634	1700	1712	1774	1848	1851	1891
1609	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1634	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1700	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**	**0**	**0**	**0**		*0,00 %*	*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1712	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**		*0,00 %*	*0,43 %*	*0,43 %*	*0,85 %*
1774	**0**	**1**	**23**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**22**	**1**	**0**	**0**	**22**	**1**	**2**	**1**	**23**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**		*0,43 %*	*0,43 %*	*0,85 %*
1848	**1**	**2**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**	**2**	**1**	**1**	**23**	**2**	**3**	**2**	**24**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**		*0,00 %*	*0,43 %*
1851	**1**	**2**	**24**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**23**	**2**	**1**	**1**	**23**	**2**	**3**	**2**	**24**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**		*0,43 %*
1891	**2**	**3**	**25**	**2**	**2**	**2**	**2**	**4**	**2**	**2**	**2**	**2**	**2**	**24**	**3**	**2**	**2**	**24**	**3**	**4**	**3**	**25**	**2**	**2**	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	

Note: P-distance= (number of different nucleotides/ Total number of analyzed nucleotides.